LOW DENSITY LIPOPROTEIN AS A TARGETED CARRIER FOR ANTI-TUMOUR DRUGS

BY

LO HOI KA ELKA

B. Sc. (Hon.), The Chinese University of Hong Kong

A Thesis Submitted in Partial Fulfilment of the Requirements for the

Degree of Master of Philosophy

in

Biochemistry

July 2001

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Prof. Fung Kwok Pui for providing me the chance to work in his laboratory as well as his continuous guidance throughout the two-year research and the preparation of this thesis.

I am grateful to Prof. Vincent Ooi of the Biology Department, The Chinese University of Hong Kong, and Freddie Kwok, his technician, for their help in the preparation of the histological sections of the hearts of the mice. I would like to thank Prof. Walter Ho of the Biochemistry Department, The Chinese University of Hong Kong, for providing the Traditional Chinese Medicine (TCM) - *Fructus Crataegi* (FC) throughout the research. I would like to thank the Research Grants Council to support this project.

I am also grateful to Dr. Sue Tsang, Mr. Andrew Chu for their guidance and technical assistance throughout the project.

Last but not least, I am grateful to all friends in Prof. Fung's Laboratory for their help, encouragement and friendship during the study.

CONTENTS

CHAPTER 5 : CONCLUSION

ABSTRACT

A major problem with current cancer chemotherapy is the lack of selectivity of antitumor drugs. Most of the drugs exert various side effects such as hepatotoxicity, cardiotoxicity and neurotoxicity on the host. In order to reduce the adverse side effects on normal cells and to enhance the anti-tumor effects, the antitumor drugs would be linked to a specific carrier, such as hormones, antibodies or enclosed in liposomes before administrations. However, a problem in *in vivo* administration of such drug-carrier complexes is their rapid clearances, induction of immunological reactions, or instabilities.

According to the previous studies, human leukaemic cells and certain tumor tissues display elevated low density lipoprotein (LDL) receptors as compared with the corresponding normal cells or tissues. Moreover, there have been a number of reports linking hypocholesterolemia and hypolipoproteinemia with various cancers. It is proposed that plasma LDL is taken up by LDL receptors on the tumor cells in the cancer patients. Therefore, LDL has been proposed as a potential carrier for antitumor drugs targeting towards the tumor cells.

In this study, a method of incorporating doxorubicin (Dox) into LDL without altering its function by mixing LDL and Dox at 37°C for 24 hours in dark was used. The cytotoxicity effects of doxorubicin (Dox) and low density lipoprotein-doxorubicin (LDL-Dox) on human hepatoma cells (HepG2) and human resistant hepatoma cells (R-HepG2) were examined. Results from the current studies suggested that in *in vitro* studies the anti-proliferative effect of LDL-Dox on HepG2 cells was

less than that of free Dox, i.e. the IC_{50} of free Dox and LDL-Dox was 1.7μM and 8.9μM for 24 hours incubation respectively, and *vice versa* on R-HepG2 cells, i.e. the IC_{50} of free Dox and LDL-Dox was 368μM and 48μM for 24 hours incubation respectively.

In order to increase the cytotoxicity effect of LDL-Dox complex on tumor cells, an alkaline extract (0.1N NaOH) from *Fructus Crataegus* (FC), a Traditional Chinese Medicine, was used to examine the possibility of elevating the expression level of LDL receptors on HepG2 cells and R-HepG2 cells. The expression level of LDL receptors was found to be higher under FC treatment in HepG2 cells but not in R-HepG2 cells as compared with control. Moreover, the cytotoxic effect of LDL-Dox on HepG2 cells was enhanced after the pre-treatment of FC, i.e. the IC_{50} of LDL-Dox with and without FC pre-treatment was 0.6μM and 8.9μM respectively.

In addition, the combined treatment of hyperthermia and antitumor drugs could enhance the anti-proliferative of free Dox and LDL-Dox on HepG2 cells and R-HepG2 cells. Hyperthermia could increase the intracellular level of free Dox in cells which led to more cytotoxicity to the tumor cells. Although hyperthermia could not increase the intracellular level of LDL-Dox in cells, the anti-proliferative effect of LDL-Dox was enhanced because hyperthermia altered the metabolic rate on the tumor cells in which the contact of Dox released from LDL-complex was increased.

In *in vivo* studies, the anti-proliferative effect of LDL-Dox was higher than that of free Dox on nude mice bearing human resistant hepatoma R-HepG2 cells.

However, the adverse effect of the LDL-Dox on the heart of tumor-bearing nude mice was alleviated comparing with that of free Dox. It was indicated that after Dox is coupled with LDL, the multidrug resistence can be circumvented.

摘要

在現今治療癌症的方法中，化學治療要面對的主要問題是抗癌药物缺乏選擇性。大部分的抗癌药物都會對癌症病人產生不同程度的副作用，例如：損害肝臟、心臟及神經系統。爲了減少抗癌药物對病者正常細胞的毒性以及增加药物的抗癌效應，科學家建議將抗癌药物與一些載體接合，當中包括激素、抗體和脂質體。不過，這些載體很快就被肝臟和胰臟的免疫細胞從血液中清除以及載體與抗癌药物的混合物並不能穩定地存在於病者體內。

根據以往的研究指出，血癌細胞上和部分癌細胞組織上的低密度脂蛋白受體(LDL-R)的數目比相關的正常細胞或組織的低密度脂蛋白受體數目爲多。同時，有很多報告指出，不同的癌症病人都會出現膽固醇和脂蛋白偏低的情況。這現象的產生是基於癌細胞是透過低密度脂蛋白受體去吸收血液中的低密度脂蛋白(LDL)作爲合成細胞膜的原料。因此科學家建議選用低密度脂蛋白作爲載體與抗癌药物結合，從而使抗癌药物更準確地運送至癌細胞，並且達到減低抗癌药物所引致的副作用。

在研究過程中，合成低密度脂蛋白阿霉素(LDL-Dox)的方法是將阿霉素(Dox)與低密度脂蛋白在攝氏 37 度和黑暗的環境下搖動 24 個小時，而這方法並沒有影響或改變低密度脂蛋白的結構和其生物活性。此次研究包括阿霉素和低密度脂蛋白阿霉素對人類肝腫瘤細胞(HepG2)及有抗药性人類肝腫瘤細胞(R-HepG2)所產生的抑制作用。在現階段的研究結果顯示，低密度脂蛋白阿霉素

對人類肝腫瘤細胞的抑制作用較阿霉素爲低：在 24 小時的治療過程中，其抑制癌細胞生長百分之五十(IC_{50})的阿霉素及低密度脂蛋白阿霉素之濃度分別爲 1.7μM 及 8.9μM；但在有抗葯性人類肝腫瘤細胞之研究結果剛好相反，即低密度脂蛋白阿霉素的抗癌能力較阿霉素爲高，在 24 小時的治療過程中，其抑制癌細胞生長百分之五十的阿霉素及低密度脂蛋白阿霉素之濃度分別 368μM 及 48μM 。

爲了更有效地提升低密度脂蛋白複合物對癌細胞的選擇性，因此選用一種由傳統中葯「北山楂」中萃取出的鹼性提取物(FC)，用以刺激人類肝腫瘤細胞及有抗葯性人類肝腫瘤細胞上低密度脂蛋白受體的表達。經過北山楂提取物處理的肝腫瘤細胞上，低密度脂蛋白受體的密度增加，但此提取物並不能增加有抗葯性旰腫瘤細胞上的受體數目。而且當肝腫瘤細胞經過北山楂提取物處理後，更可增加低密度脂蛋白阿霉素對肝腫瘤細胞生長的抑制作用：其抑制癌細胞生長百分之五十的低密度脂蛋白阿霉素經過北山楂提取物處理與否後的濃度分別爲 0.6μM 及 8.6μM。

除此之外，亦研究了高熱療法和阿霉素及低密度脂蛋白阿霉素的混合治療法對人類肝腫瘤細胞及有抗葯性人類肝腫瘤細胞之抑制作用。當癌細胞在高熱治療下，可增加癌細胞內的阿霉素濃度，繼而增加對癌細胞的殺傷力。反之，高熱治療並不能增加低密度脂蛋白阿霉素在癌細胞內的濃度，不過高熱療法能增加複合物對癌細胞的抑制作用，這是基於高熱療法能影響複合物在

癌細胞內的新陳代謝率，從而導致阿霉素較易由低密度脂蛋白複合物中釋放出來，以增加對癌細胞的殺傷力。

在植入有抗葯性肝腫瘤細胞的裸鼠模型中，低密度脂蛋白阿霉素對有抗葯性肝腫瘤細胞的抗癌能力較阿霉素爲高。而且，低密度脂蛋白阿霉素比阿霉素對裸鼠的心臟所產生的副作用爲低。這顯示出當阿霉素與低密度脂蛋白形成複合物後，既有抗癌能力，亦能克服有抗葯性的癌細胞。

Some of the results in this thesis have been published in the following paper: Andrew C. Y. Chu, S. Y. Tsang, Elka H. K. Lo and K. P. Fung. Low density lipoprotein as a targeted carrier for doxorubicin in nude mice bearing human hepatoma HepG2 cells. Life Sciences, in press.

LIST OF FIGURES AND TABLES

FIGURES

TABLES

ABBREVIATIONS

ADP	Adenosine diphosphate
AML	Melogenous leukemia
CK	Creatine kinase
CLSM	Confocal laser scaning microscopy
Dox	Doxorubicin
EPC	Egg phosphatidyl choline
FBS	Fetal Calf Serum
FC	Fructus Crataegus
FCM	Flow cytometry
FSC	Forward scatter
G-6-PDH	glucose-6-phosphate dehydrogenase
HepG2 cells	Hhuman hepatoblastoma cells
HK	Hexokinase
HMG CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDL-Dox	Low Density Lipoprotein-Doxorubicin

LDL-DRUG	Low density lipoprotein-drug
LDL-R	Low density lipoprotein receptor
LM	Light microscope
LPDS	Lipoprotein deficient fetal bovine serum
MAO	Monoamine oxidase
MDR	Mutlidrug resistance
MRP	Mutlidrug resistance-associated protein
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazoyl blue
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
PBS	Phosphate buffered saline
P-gp	P-glycoprotein
PMT	Photomultiplilier tube
PS	Penicillin-streptomycin
R-HepG2 cells	Human resistant hepatoblastoma cells
S.C.	Subcutaneously
SDOC	Sodium deoxycholate
SSC	Side scatter
TBS	Tris-buffer saline
VLDL	Very low density lipoprotein

CHAPTER 1 : INTRODUCTION

1.1. DIFFERENT TREATMENTS OF THE CANCER THERAPY

Surgery, radiotherapy, and chemotherapy are all effective treatments for cancer and they have been used alone or in a combined treatment. Surgery and radiotherapy can often eradicate primary or localized disease but may ultimately fail because of the metastasized characteristic of the cancer. In such instances, if chemotherapy is used properly, it will control or eliminate metastatic disease and reduce mortality. When chemotherapy combined with surgery or radiotherapy or both, it is usually called an adjuvant therapy. Moreover the adjuvant therapy can increase the survival rates for a number of solid tumors that were formerly treated by only one therapy.

1.2. THE SIDE EFFECTS OF CANCER TREATMENT

1.2.1. Surgery

Surgery can be a curative therapy only when the tumor is confined to an anatomical region. It is a way of removing a visible tumor to cure the disease. Surgery can relieve pain and treat complications, which are causing uncomfortable symptoms. Moreover, it would increase the effectiveness of radiation and

chemotherapy. But surgery would remove an area of normal-looking tissue surrounding the tumor to ensure that no cancer cells remain in the immediate vicinity. Also, there is a high risk for old people since it might cause death.

1.2.2. Radiotherapy

Radiotherapy is a method using the high energy to damage the cancer cells' DNA in order to make them less able to reproduce. Since cancer cells divide more quickly than those of healthy cells, they are more vulnerable to radiation. Also some tumors are more susceptible to radiation, such as nasopharyngeal carcinoma.

Although radiotherapy could take a part in treatment of cancer, some side effects often occur, such as poor appetite and skin problems. Furthermore, radiodamage does not distinguish between cancer cells and healthy proliferative cells, so the patients need special protection during the therapy.

1.2.3. Chemotherapy

Chemotherapy is a systemic treatment because it treats cancer which is growing inside the body. There are more than fifty anticancer drugs now in use for this therapy. Moreover, the anticancer drugs could destroy rapidly dividing cells or prevent them from proliferation. The most common use for chemotherapy is the adjuvant therapy because of the enhancement of the other treatment effectiveness (Table 1.1).

But most of the anticancer drugs are lack of selectivity between cancer cells and normal cells, so they may lead to severe toxic effects on normal cells (Table 1.2). Moreover, after a long term treatment, the cancer cells would be developed into multidrug resistant cancer cells.

Table 1.1: Diseases in which chemotherapy have contributed to a life-span approaching normal life expectancy (Johnson *et al.*, 1975).

Disease	Regimen
Burkitt's lymphoma	Cyclophosphamide
Choriocarcinoma	Methotrexate; actinomycin D
Acute lympocytic leukemia of childhood	Vincristine and prednisone for induction; methotrexate, 6-MP for maintenance; vincristine, prednisone, 6-mercaptopurine, methotrexate
Hodgkin's disease	Nitrogen mustard, vincristine, procarbazine and prednisone
Lymphosarcoma	Cyclophosphamide, vincristine and prednisone
Embryonal teaticular carcinoma	Actinomycin D, mithramycin, vincristine, methotrexate, bleomycin, vinblastine and *cis*-platinum
Wilm's tumor	Surgery, radiotherapy, actinomycin D and vincristine
Ewing's sarcoma	Radiotherapy, vincristine and cyclophosphamide
Rhabdomyosarcoma	Surgery, radiotherapy, vincristine, cyclophosphamide and actinomycin D
Retinoblastoma	Radiotherapy and cyclophosphamide

Table 1.2: Clinical undesirable effect of anticancer drugs.

Tissue or system affected	Toxic effects
Bone marrow	Leukopenia and lyphocytopenia with an increased risk of infection or activation of quiescent infection; Immunosuppression; Thrombocytopenia leading to hemorrhage; Anemia.
Digestive tract	Oral ulceration; Intestinal ulceration, diarrhea.
Hair root	Alopecia.
Gonads	Menstrual irregularities, ammenorrhea; infertility. Impaired spermatogenesis; sterility.
Tissues undergoing repair (surgical wounds, etc)	Impaired healing.

1.3. THE CHARACTERISTICS OF DOXORUBICIN (DOX)

1.3.1. The structure of Dox

Doxorubicin (Dox) is one of the prototypes of anthracycline anticancer antibiotic with anti-tumoral activity which is isolated from strains of *Streptomyces peucetius*. Dox is currently in widespread clinical used. The chemical structure of these antibiotics includes an aglycone chromophore with four fused rings and an amino sugar, daunosamine (Fig. 1.1).

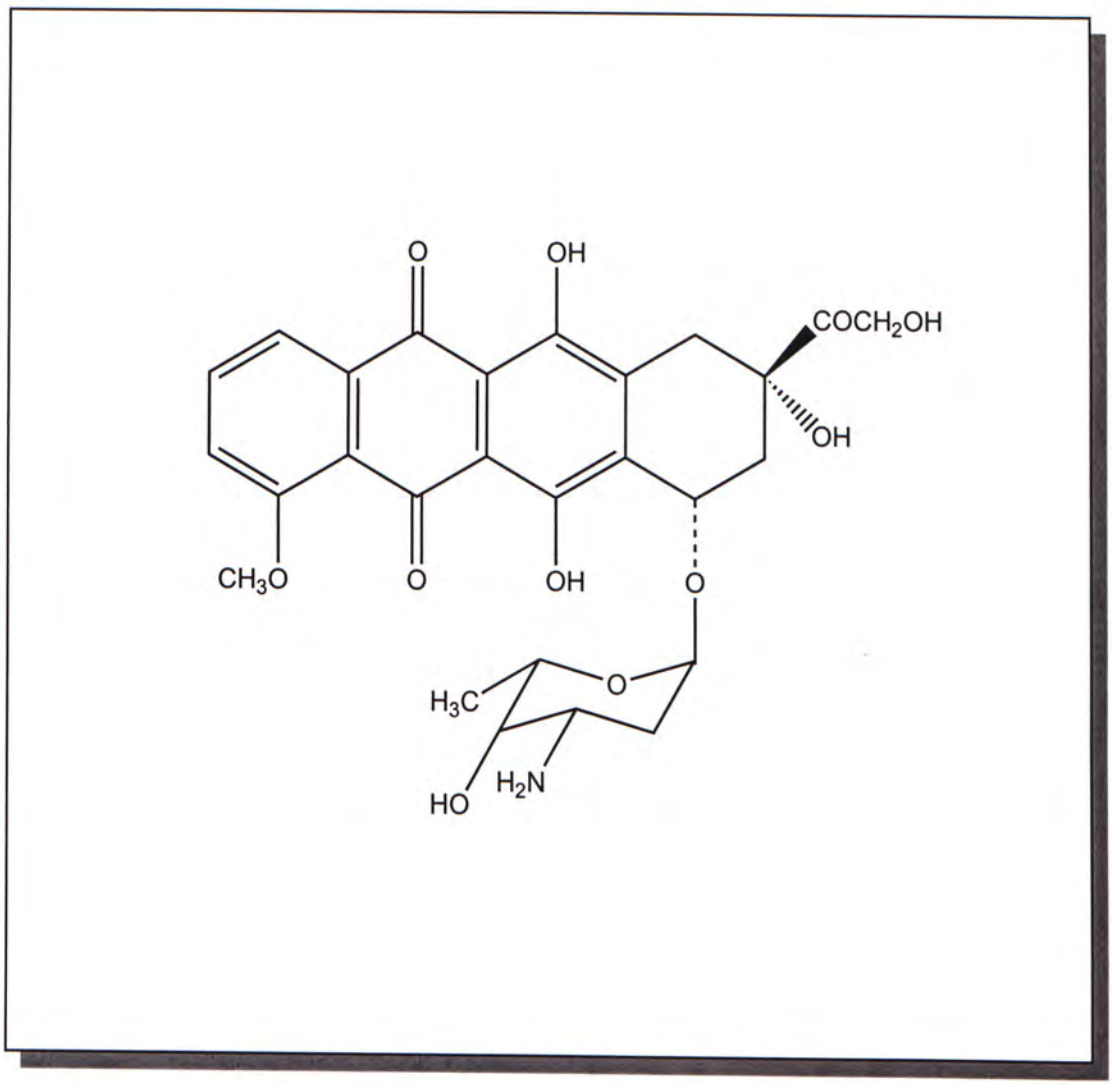


Fig. 1.1. Structure of Doxorubicin.

1.3.2. The actions of Dox

Doxorubicin (Dox) is a common and powerful anticancer drug in chemotherapy. It can treat a wide variety of solid tumors, such as carcinoma of liver, breast, lung; soft tissue sarcomas and many cancers of blood. Moreover, the multiple potential mechanism actions of anthracycline include inhibition of DNA topoisomerase I (Fogleson *et al.*, 1992) and II (Tewey *et al.*, 1984), inhibition of helicases (Bachur *et al.*, 1992), generation of free radicals through the monoelectronic reduction of the anthracyclinic ring (Bachur *et al.*, 1977), altertion of membrane structure and function (Koseki *et al.*, 1991), and exhibition of endonucleolytic clevage (Ling *et al.*, 1993).

1.3.3. The adverse side effect of Dox

Although Dox is currently used in clinical treatment, the limitation of Dox is that it may cause a potentially lethal and dose-dependent congestive heart failure which finally results in cardiomyopathy. The mechanism of Dox-induced cardiomyopathy is unclear, but most of the evidence suggested that free radicals mediated damage are involved. The chemical structure of Dox is prone to the generation of free radicals (Sinha *et al.*, 1987), and the cellular injury is caused by oxidative stress (Rosen *et al.*, 1990). When the oxidative stress is increased, it may lead to a variety of subcellular changes in the myocardium, such as the slow loss of myofibrils and vacuolization of myocardial cells. In addition, the administration of Dox is associated with the decrease in the presence of the endogenous antioxidants

which are responsible for the scavenging of free radicals (Singal *et al.*, 1995). Thus, the decrease of antioxidants and the increase of oxidant (free radicals) will increase the oxidative stress, which will result in the development of cardiomyopathy and heart failure (Singal *et al.*, 1997).

In addition, the immediate side effects of Dox treatment are reversible or clinically manageable, such as myelosuppression, nausea, vomiting and arrhythmia (Lefrak *et al.*, 1973).

1.4. THE RATIONALE OF USING LOW DENSITY LIPOPROTEIN (LDL) AS A TARGET CARRIER IN CANCER THERAPY

1.4.1 The correlation between cholesterol and cancer

There have been a number of reports which found that hypocholesterolemia and hypolipoproteinemia are usually found in patients bearing various cancers, such as leukaemia (Alexopoulos *et al.*, 1987). Several epidemiological studies have shown a correlation between low plasma cholesterol levels and the appearance of cancers (Feinleib M, 1983). However, it is unclear whether low plasma cholesterol is a risk factor for the development of malignancy or secondary to the existence of cancer (Vitols *et al.*, 1991). Regarding the risk factor for the development of malignancy, one factor may be an increase in the biliary

sterols excretion due to hypocholesterolemia which could then be metabolized by bacteria in the gut in order to increase the production of carcinogenic sterols (Reddy *et al.*, 1981). A number of studies in patients with different cancers demonstrated that low serum cholesterol levels were usually found in their blood samples (Cambien *et al.*, 1980).

In 1939, Muller (1939) noted that patients with leukaemia commonly exhibited hypocholesterolaemia. Some reports showed that most of the acute myelogenous leukemia patients (AML) have higher low density lipoprotein receptor (LDL-R) activity of leukaemic cells when compared with those from the healthy subjects (Ho *et al.*, 1978). It may be due to the increased uptake of cholesterol by leukaemic cells. These reports also indicated an inverse correlation between cholesterol concentration and the activity of low density lipoprotein receptors in leukaemic cells. The reason for the higher LDL receptor activity in leukaemic cells remains unknown, and may be due to the enhancement of membrane synthesis by the proliferating cells (Klock *et al.*, 1979). During chemotherapy, the serum cholesterol levels increase concomitantly with the decrease of the number of leukaemic cells.

In the recent studies of the prostatic carcinoma, as the cancer patients are in the metastasis phase, their serum cholesterol levels are significantly lower than that in patients who are not in the metastasis phase (Henriksson *et al.*, 1989).

In addition, a case report demonstrated that a patient bearing adrenal adenoma exhibited severe hypocholesterolemia. The increased of LDL receptor

activity and the uncontrolled uptake of LDL by the tumor cells are the result of the hypocholesterolemia. But the serum cholesterol levels rose dramatically after the resection of the adrenal tumor in this patient (Nakagawa *et al.*, 1994).

1.4.2. Low density lipoprotein (LDL) as a target carrier

The major problem with the current cancer chemotherapy is that the anticancer drugs are often limited by their inability to discriminate between normal cells and cancer cells, inducing severe toxic effects on normal cells rather than on cancer cells. One way to enhance the specificity of the anticancer drugs is to link them to a carrier which is specifically taken up by the tumor cells. One carrier that has recently stimulated interest is low density lipoprotein (LDL) because the tumor cells display elevated low density lipoprotein receptor mediated uptake of LDL comparing with the normal cells (Fig. 1.2).

LDL is a component of plasma whose physiological function is the transport of cholesterol. LDL can administer the highly lipophilic compounds. Moreover, the core of LDL provides spacing for drugs sequestration in order to isolate them from serum enzyme and water (Vitols *et al.*, 1990). LDL is a physiological carrier that is not easily cleared by the reticuloendothelial system. The serum half-life of anticancer drugs can be prolonged when they are incorporated into LDL (De Smidt *et al.*, 1990). Also, LDL can be internalized and degraded by LDL receptor pathway in cancer cells. This highly effective process could lead to different

pharmacological effects, such as circumvention of some drug resistance mechanism (Iwanik *et al.*, 1984).

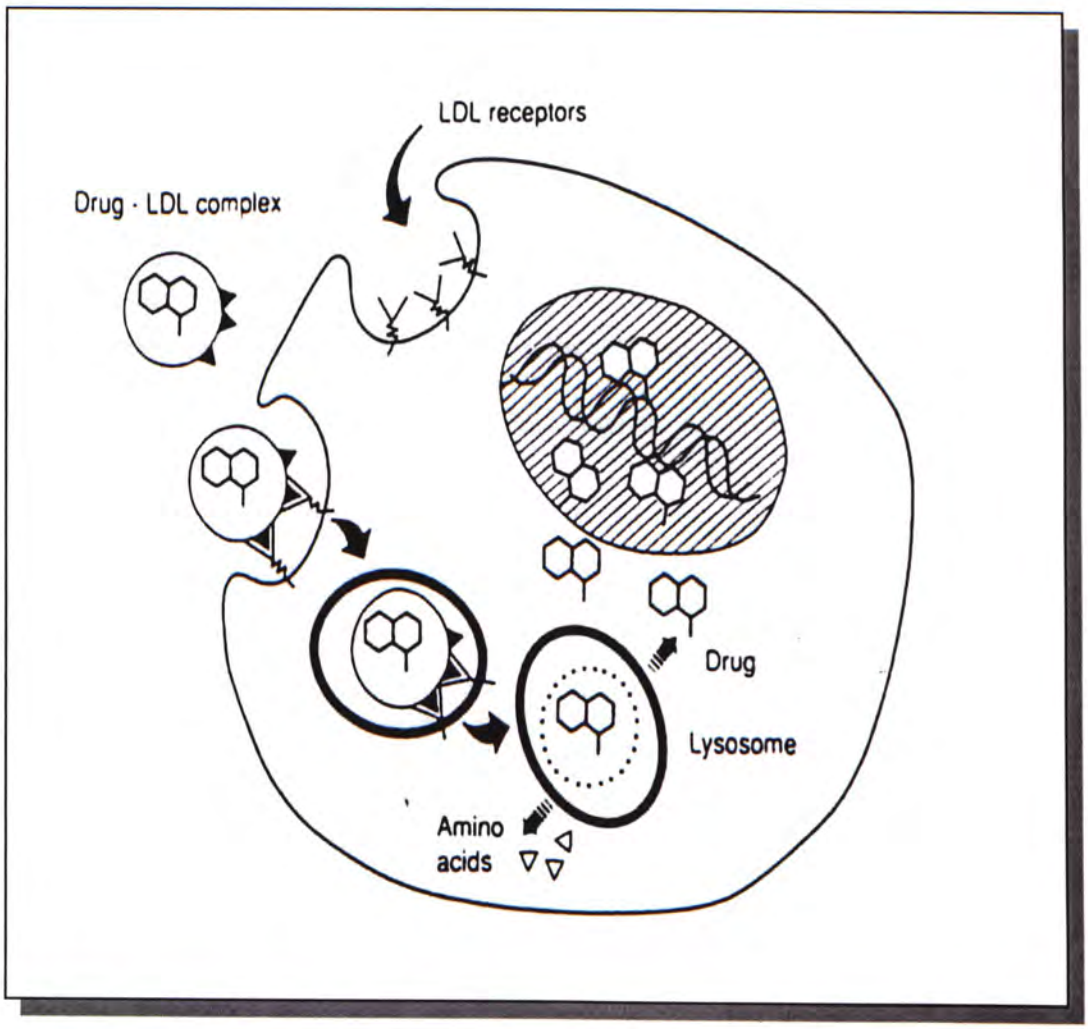


Fig. 1.2. The process for the receptor-mediated endocytosis of a lipophilic drug incorpated into LDL.

1.4.3. The down and up regulation of LDL receptors

Although it is important to deliver LDL-drugs only to the tumors and exclude them from reducing the normal tissues, some tissues and organs such as liver and adrenal could also compete to uptake the drug complex. Thus, protection of these tissues and organs is desirable when LDL-drugs are administered to the patients.

Some reports found that after feeding the tumor bearing mice with sodium taurocholate and hydrocortisone sodium succinate, the uptake of LDL by liver and adrenal was reduced, but the uptake of LDL by the tumor did not have a significant reduction (Hynds *et al.*, 1984). A report demonstrated that saturated fats, cholesterol with hydrogenated coconut oil and fasting could down-regulate the LDL receptors on normal cells, but not the tumor cells (Shimano *et al.*, 1988).

On the other hand, it is crucial to elevate the LDL receptors on the tumor cells, but not on normal cells in order to increase the uptake of LDL-drug complex. There are some chemicals and hormones which can up-regulate the LDL receptors in tumor cells, such as taurine (Zouhair *et al.*, 1987), insulin (Nagels *et al.*, 1997), estrogen, thyroid hormone and glucagons (Parini *et al.*, 1995), troglitazone (Rayyes et al., 1998) and *Fructus Crataegus* (Wilmar S, 1960).

1.4.4. The characteristics of *Fructus Crataegus* (FC)

Fructus Crataegus (FC) is the crataegus pinnatifida Bunge. var. major N. E. Br. Reports showed that the extraction of FC can lower the serum cholesterol, especially low density lipoprotein (LDL) and very low density lipoprotein (VLDL) (Chu, 1988). Also FC can increase the activity of superoxide dismutase (SOD) and reduce the activity of monoamine oxidase (MAO). The components of FC such as hyperoside, epicatechin, rutin and quercitin can reduce the serum cholesterol level (Sit *et al.*, 1985).

In this project, the crude extract of FC is used to investigate the up-regulation of LDL receptor on tumor cells. The crude extract of FC consists of eight pure compounds; they are ursolic acid, protocatechuic acid (Fig. 1.3), quercitin, epicatechin, hyperoside, isoquercitrin, chlorogenic acid and rutin (Fig. 1.4).

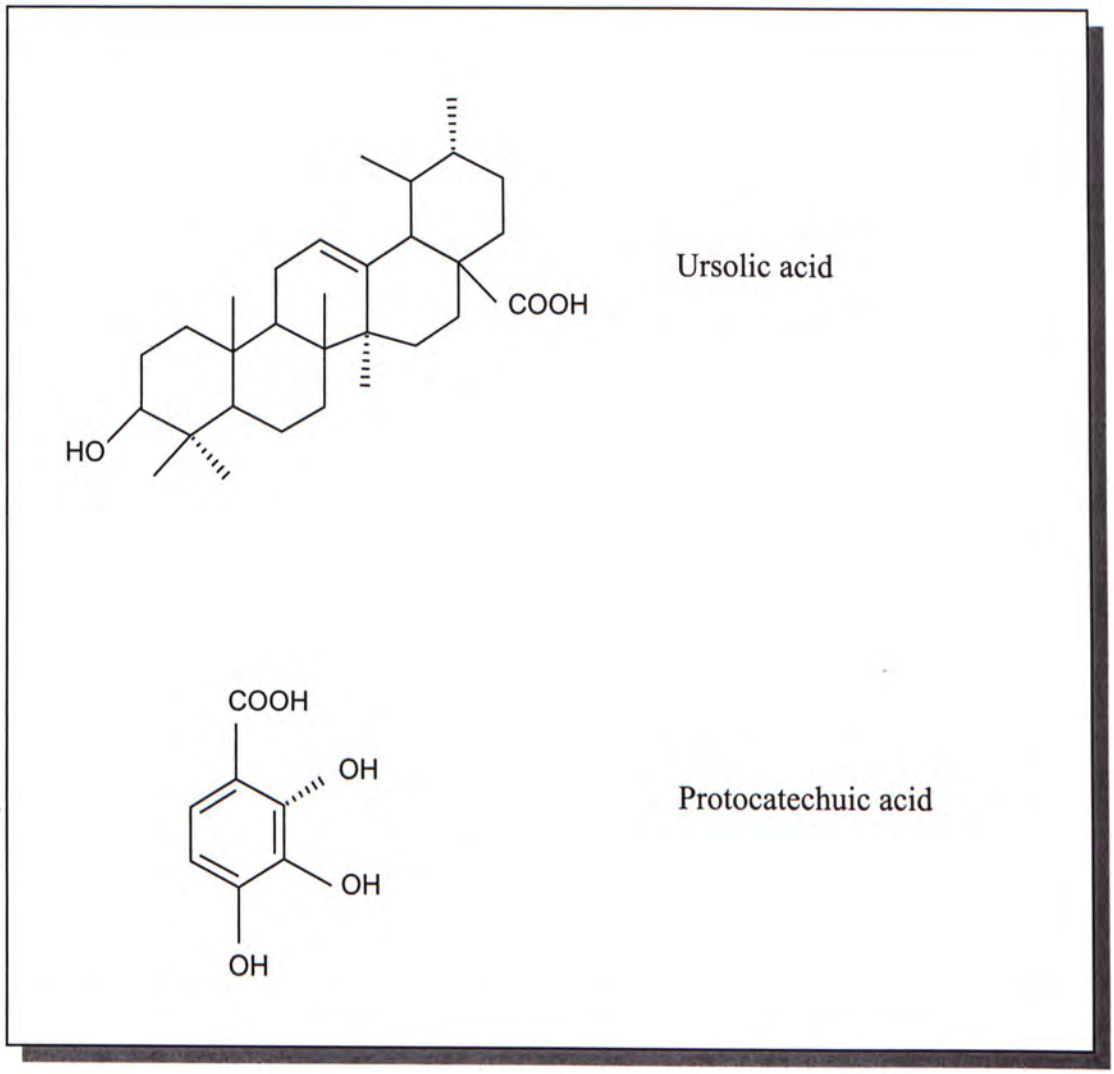


Fig. 1.3. Chemical structure of *Fructus Crataegus* (FC).

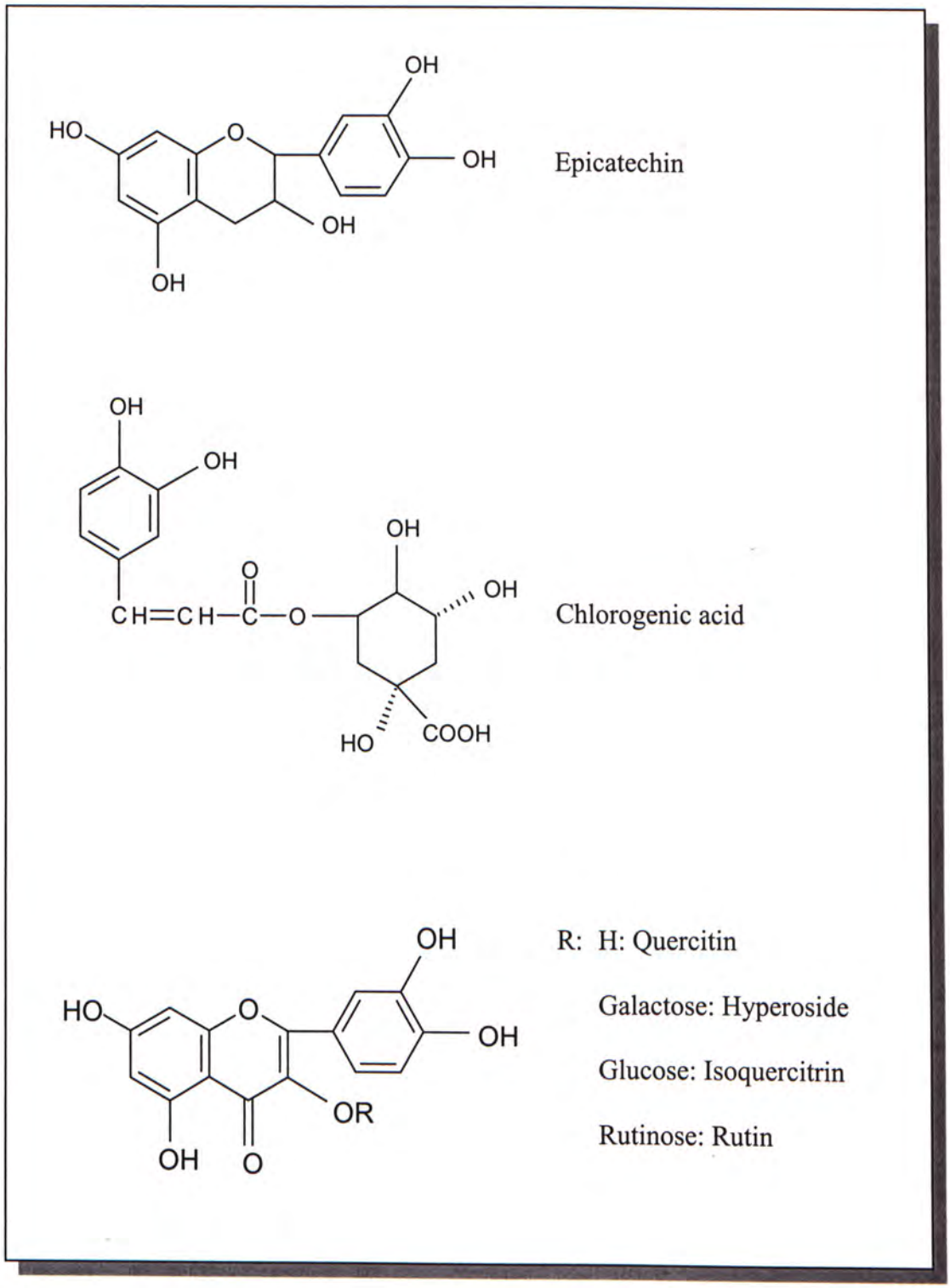


Fig. 1.4. Chemical structure of *Fructus Crataegus* (FC).

1.5. DIFFERENT METHODS OF THE PREPARATION OF THE LOW DENSITY LIPOPROTEIN-DRUG (LDL-DRUG)

In order to kill tumors with drugs that are targeted to the LDL receptors, the drugs must first be bound to LDL. When this drug-complex is traveling in the blood, it should not be dissociated. Moreover, its cytotoxicity should be restored after entering into the tumor cells.

The first published paper concerning the procedure of LDL-drug synthesis was reported by Krieger *et al.*, (1979), in which hydrophobic compound is incorporated into native LDL. The LDL was isolated, lyophilized in the presence of potato starch and the core of LDL was delipidated by heptane extraction. Then the heptane-extracted LDL was reconstituted by adding the drug which was dissolved in the nonpolar solvent. Finally, the solvent was evaporated and the reconstituted LDL was replaced with aqueous buffer. Although the carrying power of the reconstituted LDL was high, the entire core is replaced which may lead to leakage of the drug from reconstituted LDL or failed to reconstitute altogether. Moreover, the creation of aggregated reconstituted LDL would by rapidly cleared by the reticuloendothelial system.

In the method of Masquelier *et al.*, (1986), the LDL was lyophilized in the presence of sucrose as protecting agent and the core of LDL was not discarded.

The pharmacokenetics of reconstituted LDL was found to be more closely to the native LDL, but the drug carrying power was lower when comparing with the method of Krieger *et. al.* (1979). Also, this method was easier for reconstitution without aggregation.

In the method of Lundburd (1984), LDL is delipidated by a detergent (sodium deoxycholate, SDOC), then the apoprotein B is resonstituted with the drug and egg phosphatidyl choline (EPC) into a microemulsion. The LDL-drug was isolated by ultracentrifugation through a sucrose gradient. Moreover, the yield and carrying powder of the LDL drug from this method was higher than that of the LDL-drug formed from other methods. Also, the structure of the LDL-drug comlplex was similar to that of the native LDL.

In the method of Rudling et al., (1983), the procedure was very simple and that LDL was singly mixed up with the drug. The LDL was incubated with an excess amount of the drug, then using the gel filtration method to separate the LDL-drug and free drug from the mixture. In addition, the drug can be chemically linked to LDL and the leakage will not occur in this method. But the carrying power is limited and the cytotoxicity of the LDL-drug may be lower unless it is released efficiently after endocytosis (Vitols *et al.*, 1984).

The method used in this project is the aforesaid simple mixing technique to incorporate doxorubicin (Dox) into LDL (Rudling *et al.*, 1983). The excess amount of Dox was used to incubate with LDL at 37°C for 24 hour and free

Dox was separated from LDL-Dox complex by gel filtration. In addition, Rudling *et. al.* (1983) and Iwanik *et. al.* (1984) have also used this method to incorporate other drugs into LDL. The advantage of this method is that the procedure is very simple and does not alter the structure of the LDL, thus the pharmacokenetics of LDL-drug complex is very close to that of the native LDL.

1.6. THE CHARACTERISTICS OF LOW DENSITY LIPOPROTEIN (LDL)

Cells may obtain cholesterol in two ways: by endogenous synthesis through *de novo* pathways or by the uptake of cholesterol-containing particles, namely lipoproteins, from the environment. The major cholesterol-carrying particle in human plasma is low density lipoprotein (LDL). In healthy individuals, the half-life of the LDL in plasma is about 2 to 3 days.

1.6.1. The structure of LDL

LDL is a large spherical particle that has a molecular weight of approximately 3 x 10^6 Dalton and the diameter is 22nm (Krieger *et al.*, 1979). In each LDL particle, the core contains about 1500 molecules of cholesterol esterified with long-chain fatty acids such as oleate or linoleate. A shell of phospholipids and unesterified cholesterol surrounds the highly hydrophobic core. The phospholipid is mixed with unesterified cholesterol, presumably as a stabilizer and also a single

molecule per LDL particle of apoprotein B which bind to the specific cell surface receptor (Fig. 1.5).

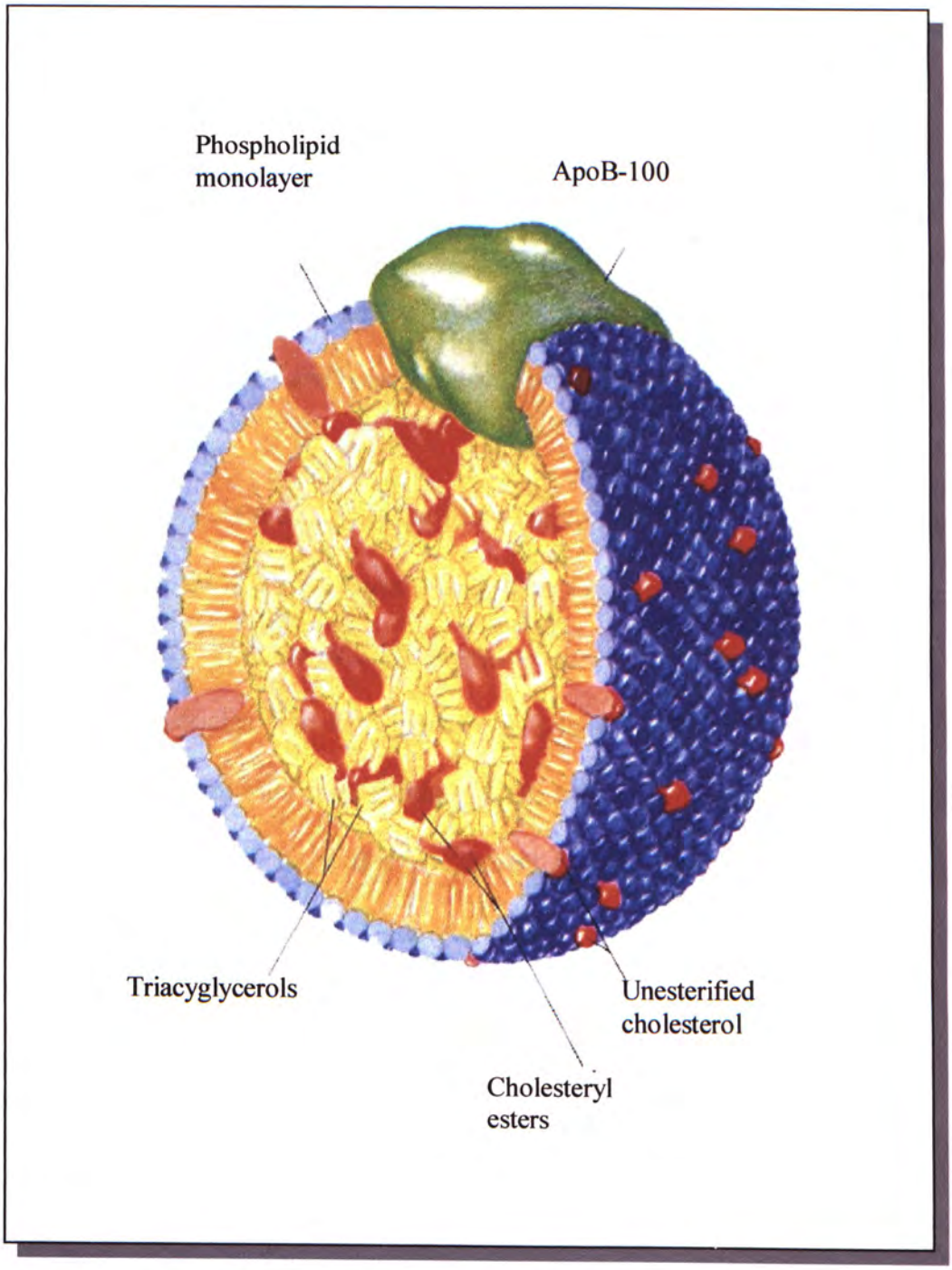


Fig. 1.5. Structure of low density lipoprotein (LDL) (Lehninger, 1993)

1.6.2. The metabolic pathway of LDL in human bodies

Cells acquire cholesterol for membrane synthesis primarily through the adsorptive endocytosis of plasma LDL. The initial step in this uptake process is the high affinity binding of LDL to the cell surface receptors --- LDL receptor followed by internalization and lyosomal degradation of the lipoprotein particle.

Since each LDL particle has a single molecule of apoprotein B that can interact with the LDL receptors. After LDL binds to the LDL receptors, the bound LDL is taken into the cells by incorporation into endocytotic vesicles. Then the interiorized vesicles fuse with lysosomes. Lysosomal enzymes hydrolyze the protein and cholesterol ester components of LDL as the apoprotein B is degraded to amino acids and the liberated free cholesterol is transferred to cell membranes (Brown *et al.*, 1975). Cellular cholesteryl ester formation is stimulated and cholesterol synthesis is suppressed by the mechanisms involving the membrane-bound enzymes, fatty acyl CoA: cholesteryl acyltransferase, and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase). The entire system functions as a means of obtaining controlled access to cholesterol brought to the cell as plasma LDL. The system controls LDL cholesterol entry by controlling synthesis of LDL receptors via a feedback loop (Fig. 1.6).

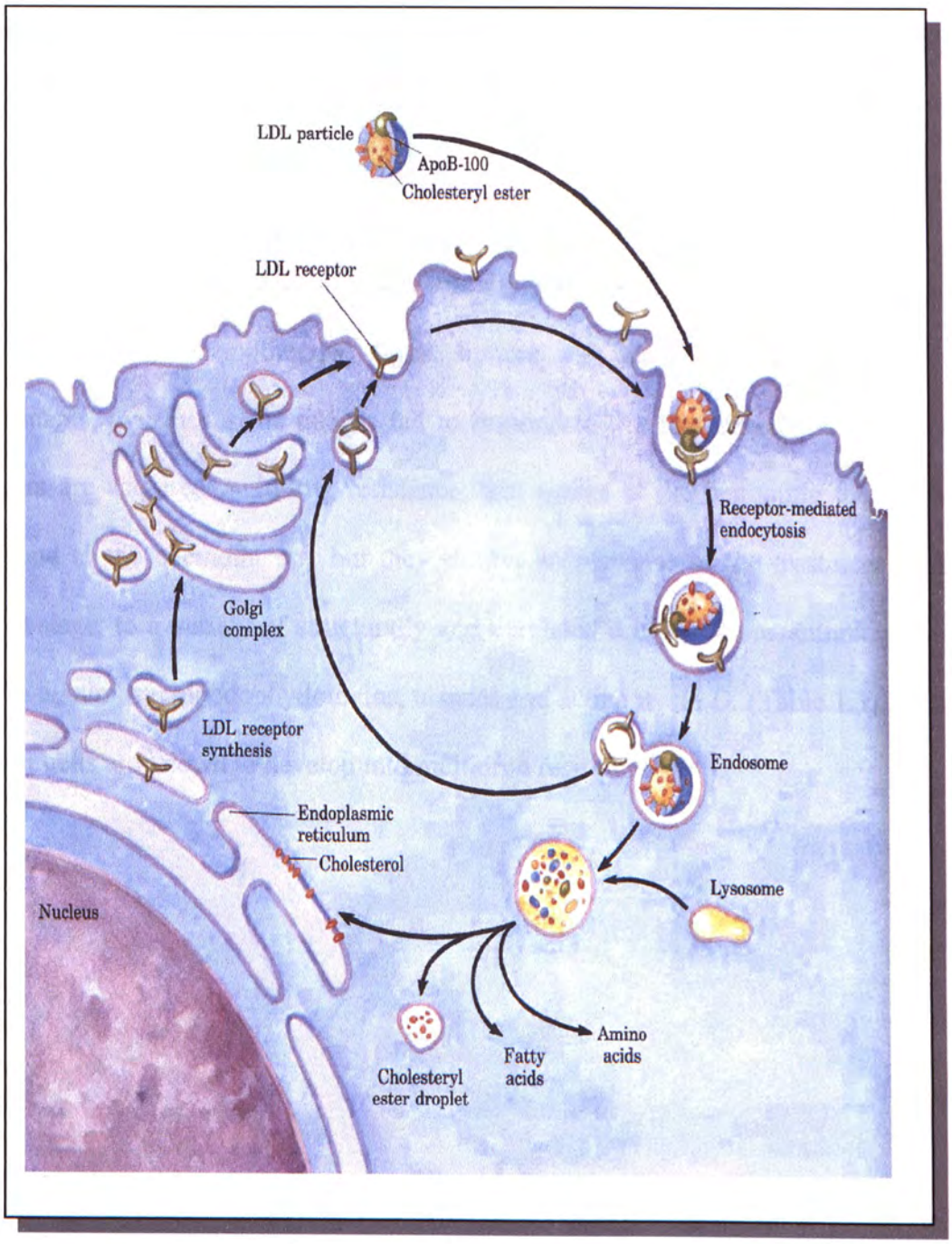


Fig.1.6. The metabolic pathway of low density lipoprotein in human bodies (Lehninger, 1993).

1.7. THE MULTIDRUGS RESISTANCE IN TUMOR CELLS

Resistance to the anticancer drugs in cancer cells is one of the major problem of the chemotherapy. Some tumors are intrinsically resistant to the treatments, that means the tumors fail to respond to the first chemotherapy given. Others are acquired multidrug resistance, that means at the beginning the tumors respond to the chemotherapy but they exhibit no response of the treatment at the latter stage, to a variety of structurally and unrelated drugs, such as anthracyclines, vinca alkaloids, epipodophyllotoxins, taxanes and actinomycin D (Table 1.3). These tumor cells are known to develop into multidrug resistance.

Table 1.3: Spectrum of drugs in multidrug resistance.

Anthracyclines	Doxorubicin
	Daunorubicin
	Epirubicin
Vinca alkaloids	Vincristine
	Vinblastine
Epipodophyllotoxines	Etoposide
	Teniposide
Taxanes	Docetaxel
	Paclitaxel
Other anticancer drugs	Actinomycin D
	Topotecan
	Mitthramycin
	Mitomycin C

1.7.1. The mechanism of multidrug resistance

The potential resistance mechanisms are the mutlidrug resistance (MDR) phenotype and mutlidrug resistance-associated protein (MRP). The MDR phenotype is related to a membrane-associated P-glycoprotein (P-gp) encoded in human cells by the MDR 1 gene which is located at the chromosome 7 (Roninson *et al.*, 1986), and confers cross-resistance to a broad spectrum of drugs, such as anthracyclines. In addition, the MRP pump, which is the membrane-associated efflux mechanism, is able to extrude drugs from the cells against the concentration gradient. Moreover, MRP action is not significantly affected by the reversing agents, such as verapamil or cyclosporin A (Barrand *et al.*, 1993).

In addition, the drug resistance occurs through different mechanisms: increasing the activity of enzymes, such as glutathione-S-transferase (Morrow *et al.*, 1990), glutathione peroxidase (Kramer et al., 1988), superoxide dismutase (Doroshow *et al.*, 1991), and altering the activity of topoisomerase II (Kim *et al.*, 1991) or hexose phosphate metabolism (Yeh *et al.*, 1987).

1.7.2. The structure of P-glycoprotein

P-glycoprotein (P-gp) is a member of the large ATP-binding cassette superfamily of transport protein also called traffic ATPases (Pietro *et al.*, 1999). P-gp is a 170-kDa plasma membrane protein and an integral membrane protein of 1280 amino acids arranged into two homologous halves, each containing six

transmembrane domains and an ATP binding domain, separated by a flexible linker polypeptide (Jone *et al.*, 1998). The pore size of P-gp is 5nm. ATP binding/utilization and hydrolysis appear to be essential for the proper functioning of P-gp, including the role as a drug transport (Horio *et al.*, 1988) (Fig. 1.7).

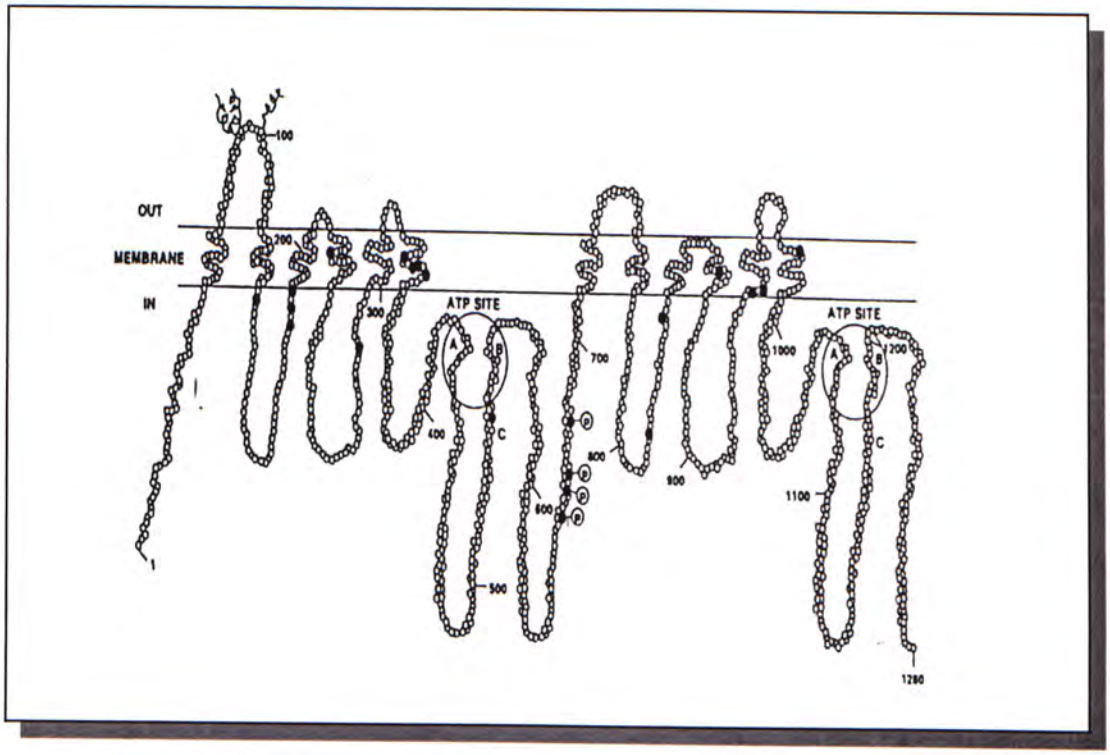


Fig. 1.7. Structure of P-glycoprotein (Gottesman et al., 1988).

1.7.3. The mechanism of P-glycoprotein

Although several models for P-glycoprotein (P-gp) function have been proposed, there is still no clear understanding at a molecular level of how the multidtrug transporter lowers intracellular accumulation of anticancer drugs.

P-glycoprotein (P-gp) exists in several normal tissues in which it probably has a physiological role in elimination of xenobiotics and in protecting important tissues from some endogenous metabolites when they are in the blood (Cardo *et al.*, 1989). Cancer cells seem to exploit this function to protect themselves from many cancer drugs. Therefore, P-gp could exert active drug efflux out of mutlidrug resistant cells in order to reduce accumulation of the drugs, which are prevented from reaching their cellular targets.

Roepe *et al.* (1997) proposed that overexpression of the P-gp could alter the electrical membrane potential and intracellular pH and other biophysical characteristics of the tumor cells. These alterations in the biophysical parameters of the tumor cells then perturbed the intracellular level of anticancer drugs. Thus, P-gp indirectly promotes decreased intracellular drug accumulation.

Gottesman (1988) proposed a pump model of P-gp to explain the function of the drug transporter. The energy of ATP hydrolysis by P-gp is utilized for the removal of drugs from cell membranes and cytoplasm analogous to the ion-translocating pumps. After the pump recognizes substrates through a complex

substrate recognition region, it would directly pumps drugs out of the cell by using molecular mechanisms, but the mechanisms are still unknown.

1.8. COMBINED TREATMENT WITH HYPERTHERMIA

Combined treatment is the combination of two or more individual treatments in order to increase the effectiveness of the treatments; also the toxic side effect of each individual treatment can be minimized. The effects of combined treatment can be additive, i.e. the total effect is equal to the summation of the individual effects. It can also be synergistic, i.e. the combined effect is larger than the summation of the individual effects. However, it may be partially additive, i.e. the combined effect is less than the summation of the individual effects. This may be due to the individual agents exhibit their effects by the same mechanism.

Hyperthermia is commonly used to combine with chemotherapy or radiation as an adjunct cancer therapy. It is because the amount of anticancer drugs or the dosage of radiation can be reduced when hyperthermia is used together. The adverse side effects from chemotherapy and radiotherapy also can be reduced when combined with hyperthermia.

The biophysical effects of hyperthermia remain unknown, but it may denature membrane protein (Lepock *et al.*, 1982), increase neovascular permeability (Gnant *et al.*, 1999), and perturbation of multimolecular complexes (Calderwood *et*

al., 1983). Tumor cell inactivation is time and temperature dependent which starts at 40 - 41°C. Moreover, human tumor cell lines are more sensitive to mild hyperthermia, i.e. around 41 - 42°C (Armour *et al.*, 1993).

The solid tumor is sensitive to hyperthermia because of the susceptible microenvironment with low pH, low oxygen tension, low glucose and the loss of adaptive vasodilatation in response to heat.

As the chemotherapy is combined with hyperthermia, the antitumor effect of various anticancer drugs is enhanced, such as doxorubicin, mitomycin C, cisplatin. It may be due to the increase of cell membrane permeability, the alteration of drug transport activity and the alteration of cell metabolism. Moreover, hyperthermia can decrease tumor tissues interstitial fluid pressure, enhance convection-driven macromolecular drug delivery (Leunig *et al.*, 1992). But hyperthermia could induce the expression level of multidrug resistance gene (MDR 1), consequently the multidrug-resistant phenotype is developed.

In addition, when hyperthermia is combined with radiotherapy, it also enhances the effectiveness of radiotherapy on the tumors. It can be explained that hyperthermia is cytotoxic to the cells which are under an environment with low partial pressure of oxygen and pH (Overgaard *et al.*, 1989). Also when the hyperthermia is applied at a mild temperature, it can induce the reoxygenation of the cancer cells in order to make them more sensitive to radiation (Shakil *et al.*, 1999).

1.9. AIM OF THE STUDY

Antitumoral drugs always exhibit adverse side effects on normal cells because of the lack of the selectivity. Moreover, the drug resistance is usually a problem in chemotherapy. So there has been an urgent need to develop a target carrier for antitumoral drugs in order to increase drugs' specificity to tumor cells, decrease their toxicity to normal cells and circumvent multidrug resistance in tumor cells.

The aim of this study was to investigate whether low density lipoprotein (LDL) can be used as a target carrier because the current studies demonstrated that the tumor cells express high density of low density lipoprotein receptor (LDL-R) on the membrane of the tumor cells. Thus this approach may be more specific to deliver antitumoral drugs to the tumor cells in order to decrease the cytotoxic effect on normal cells. Also it may overcome the efflux of antitumoral drugs by P-glycoprotein on resistant cell lines. Moreover, *Fructus Crataegus* (FC) is used to investigate the up-regulation of LDL-R on tumor cells in order to enhance the accumulation of LDL-drug complexes. Also using combined treatment strategy to investigate whether hyperthermia can enhance the cytotoxic effect of LDL-drug complexes. The cell line used in the study is human hepatoblastoma cells (HepG2 cells) and human resistant hepatoblastoma cells (R-HepG2 cells). For the investigation in this study, doxorubicin (Dox) was used because Dox is a common antitumoral drug in chemotherapy.

CHAPTER 2 : MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Animals

Protocols relating to animal studies in this thesis have obtained the approval of the Animal Research Ethics Committee, The Chinese University of Hong Kong. Nude mice aged 6 - 8 weeks were bred at the Laboratory Animal Service Center of The Chinese University of Hong Kong under pathogen-free condition. Nude mice were kept in autoclaved cage with polyester fiber filters to avoid contact with the pathogens. All the animal diet (PICO LAB$^{@}$ Rodent Diet) and tape water were autoclaved before feeding to nude mice *ad libitum*.

2.1.2. Buffers

Phosphate buffered saline (PBS)

It was prepared by mixing of 136mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4 and 8mM Na_2PO_4. The solution was titrated to pH7.4 and steriled by autoclave and stored at room temperature.

Normal saline

It was prepared by dissolving 9g of NaCl into 1 liter of the double distilled water and steriled by autoclave. It was stored at 4°C.

Tris-buffer saline (TBS)

It was prepared by dissolving 1.2114g Tris and 8.766g NaCl into 1 liter of double distilled water. The solution was titrated to pH 8.0 and stored at room temperature.

10% buffer formalin

It was prepared by mixing of 900ml double distilled water, 100ml formalin, 8.5g NaCl, 4g $NaH_2PO_4 \bullet 2H_2O$ and 6.5g Na_2HPO_4. The solution was stored at room temperature.

Harris hematoxylin

It was prepared by mixing of hematoxylin Power, sodium iodate, ammonium atum, ethanol and double distilled water. The solution was stored at room temperature.

0.5% Eosin, Aqueous

It was prepared by mixing of 200ml double distilled water, 1g eosin y, acetic acids and 2 – 3 drops of glacial. The solution was stored at room temperature.

Scott's tap water

It was prepared by dissolving 2g potassium carbonate, 20g magnesium sulphate into 100ml double distilled water and the solution was stored at room temperature.

1% Acid Alcohol

It was prepared by mixing of 1ml conc. HCl and 99ml 70% ethanol. The solution was stored at room temperature.

2.1.3. Cell Culture Reagents

Roswell Park Memorial Institute tissue culture medium 1640 (RPMI 1640 medium)

It was purchased from GibcoBRL Life Technologies Inc. and was used for cell culture experiment. Each pack of RPMI 1640 powder medium containing phenol red, L-glutamine and 0.5mM HEPES. The medium powder was

dissolved in 1 liter of double distilled water and was supplemented with 2g of sodium bicarbonate ($NaHCO_3$). The pH of the medium was adjusted to 7.2. Finally, the medium was filtered by 0.22μm bottle-top filter which was purchased from Millipore Company.

The complete RPMI 1640 medium was added with 1% penicillin-streptomycin (v/v) and 10% fetal bovine serum (FBS) (v/v). When the cells were treated with Doxorubicin coupled with LDL, 10% Lipoprotein deficient fetal bovine serum (LPDS) was used instead of using 10% of FBS.

Fetal Calf Serum (FBS)

It was purchased from GibcoBRL Life Technologies Inc. and was stored at –20°C.

Lipoprotein deficient fetal bovine serum (LPDS)

It was purchased from Sigma Chemical Company and was stored at –20°C.

Penicillin-streptomycin (PS)

It was purchased from GibcoBRL Life Technologies Inc and was stored at –20°C.

2.1.4. Chemicals

Doxorubicin (Dox)

It was purchased from Sigma Chemical Company. A 10mg/ml stock solution of doxorubicin was made in autoclaved double distilled water and stored at –20°C. This doxorubicin was used both in cell culture and animal tests. When doxorubicin was used to couple with LDL, it was also dissolved in autoclaved double distilled water at the concentration of 10 mg/ml and stored at –20°C.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

It was purchased from Sigma Chemical Company and was dissolved in 1X PBS at the concentration of 5 mg/ml. The solution was then passed through a 0.22μm millipore filter (Millipore Company) and stored at 4°C.

Low density lipoprotein (LDL)

It was purchased from Sigma Chemical Company, and according to the manufacturer, this LDL was isolated from plasma by using the modified methods of Rudel *et. al.,* (1974) and Fellin *et. al.,* (1974). It was concentrated and dialyzed extensively against 0.15 M NaCl, 0.01% EDTA, pH 7.4 - 7.5. It was then filtered through a 0.2μm membrane. Its composition is 22% of protein and 78% of lipid. The

protein concentration varies from 5 mg/ml to 6.5 mg/ml. Its molecular weight is 3.5 x 10^6 with diameter 22nm (Burstein, M. *et. al.*, 1977).

Acidified isopropanol

It was prepared by dissolving 0.75M HCl in 90% isopropanol and stored at room temperature.

Fructus Crataegus (FC)

It was provided by Prof. Walter Ho's Laboratory, Department of Biochemistry, The Chinese University of Hong Kong. It was dissolved in double distilled water and the solution was titrated to pH 7.0. Then the solution was boiled at water bath for 15 minutes for the sterilization. Before using in cell culture, the solution was centrifuged at 2200x g for 5 minutes.

Antibodies

For Western blot analysis, Low Density Lipoprotein Receptor (LDL-R), primary antibody against LDL-R were purchased from Calbiochem. The secondary antibody, horse–radish peroxidase conjugated anti-mouse antibody were purchased from Amersharm Pharmacia Biotech. For P-glycoprotein analysis, primary antibody against P-glycoprotein was purchased from Calbiochem. The

secondary antibody, horse-radish peroxidase conjugated anti-rabbit antibody used was purchased from Santa Cruz Biotechnology, Inc.

2.1.5. Culture of cells

Differentiated Hepatoblastoma cell line (HepG2 cells)

It was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). According to producer, this cell line was derived from a liver tumor biopsy obtained during extended lobectomy of a 15-year-old Caucasion male from Argentina (Aden *et. al.*, 1979). The liver tumor was diagnosed histologically as a well-differentiated hepatoblastoma. Cells were maintained in RPMI 1640 medium and supplemented with 10% FBS and 1% penicillin-streptomycin (complete RPMI). They were cultured at 37°C, 5% CO_2 incubator with humidified atmosphere. Cells were grown at culture flask and kept to pass every 3 or 4 days. For every passage, medium was discarded and washed once with PBS. Cells were trypsinized. The cell suspension was collected after addition of the complete medium to stop the trypsinization. This suspension was centrifuged at 300x g for 3 min. The pellet was resuspended with complete medium. Cells at 5 x 10^4 cells/ml were passed to a new tissue culture flask.

Development of doxorubicin resistant hepatoma cell line (R-HepG2 cells)

This resistant cell line was developed in our laboratory by incubating the HepG2 cells in stepwise increased concentrations of doxorubicin. After HepG2 cells were grown to about 80% confluence, they were treated with doxorubicin at the concentration, which caused 90% of cell death with 48 hours incubation, namely 0.1μM. After incubating 24 hours with doxorubicin, the cells were washed several times with PBS and then incubated in fresh medium for another 24 hours. The cells were washed several times again with PBS and then incubated with fresh medium. This washing procedure was repeated until the cells grew to confluence. Cells were trypsinized and divided into two new flasks. After the cells grown to 80% of confluence, 0.2μM of doxorubicin was incubated with the cells. The aforesaid treatments were repeated until the cell grew at 1.2μM of doxorubicin.

2.2. METHODS

2.2.1. *In vitro* studies

2.2.1.1. LDL, doxorubicin complex formation

10 mg of doxorubicin was dissolved with 1ml of autoclaved double distilled water. After vigorous vortex, all the powder was dissolved. One ml of LDL, which contained 6.2 mg of protein, was mixed with 62μl of doxorubicin solution

which contained 0.62mg doxorubicin. The mixture was incubated in shaking air-bath at 37°C for 24 hours in dark at 800x g. The mixture was then centrifuged at 800x g for 10 minutes and loaded onto gel filtration on G25 Sephadex column, which was purchased from Amersham Pharmacia Biotech, using 0.9% saline as running buffer to separate the free doxorubicin from Low Density Lipoprotein-Doxorubicin (LDL-Dox) complex. Fractions were collected. The LDL-Dox complex so obtained was sterilized by passage through a 0.45μm acetate millipore filter, which was purchased from Millipore Company.

2.2.1.2. Determination of the concentration of LDL-Dox

After doxorubicin is coupled into LDL, it is not accurate if its concentration is determined only by reading absorbance at wavelength 480nm because it is encapsulated in the LDL. So releasing doxorubicin from LDL is important for measuring its concentration in the complex. Acidified isopropanol was used in this aspect. Dox in LDL-Dox complex was extracted by adding 780μl of acidified isopropanol into 20μl of the complex. A standard curve of the concentration of doxorubicin in acidified isopropanol versus the absorbance at wavelenght 480nm was obtained. The concentration of LDL-Dox was determined by using this standard curve.

2.2.1.3. *In vitro* cytotoxicity

The relationship between cells and drug sensitivity could be determined by MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. Firstly, cells at 2 x 10^4 cells/well, 1 x 10^4 cells/well and 5 x 10^3 cells/well were seeded into 96-well plate overnight for 24 hours, 48 hours and 72 hours respectively. Then the medium was replaced with various concentrations of Dox in RPMI 1640 with 10% FBS or LDL-Dox in RPMI 1640 with 10% lipoprotein deficient serum (LPDS). The plate was then incubated at 37°C, 5% CO_2 for different time courses treatment.

After the treatment, the medium in each well was removed and the cells were washed twice with PBS. Then 30ul of 5mg/ml MTT solution was added to each well for 2 hours at 37°C, 5% CO_2. After that, the MTT solution was discarded and 100ul of DMSO was added to each well in order to dissolve the crystal formed by the cells. These plates were incubated for 15 min at 37°C, 5% CO_2. Finally, the plates were measured by ELISA microplate reader (BIO-RAD) at wavelength 540nm.

The results were presented as mean of percentage of survival ± S.D. for the indicated number of different experiments. The percentage of survival cells was calculated by dividing the difference of the absorbance of treated cells and the absorbance of untreated cell multiplied by 100.

2.2.1.4. The cytotoxicity of the combined treatment with anticancer drugs

2.2.1.4.1. Combined treatment of hyperthermia with anticancer drugs

The relationship between cells and combined treatment of drug and hyperthermia could be determined by MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Firstly, cells at 1 x 10^4 cells/well were seeded into the 96-well plate overnight, then different concentrations of Dox in RPMI 1640 with 10% FBS or LDL-Dox in RPMI 1640 with 10% lipoprotein deficient serum (LPDS) were added as well as incubated at 43°C for 4 hours at different time interval.

For the 48 hours drug treatment, one of the approaches was that after adding the drugs, the cells were incubated at 43°C for 4 hours immediately. Then the cells were changed back to incubate at 37°C for the rest of 44 hours after adding drugs.

Another approach was that, the cells were incubated for 22 hours after adding the drug, then the incubation temperature was changed from 37°C to 43°C and the incubation will be continued for 4 hours. Then cells were changed back to incubate at 37°C until 48 hours after adding the drugs.

After the treatment, the medium in each well was removed and the cells were washed twice with PBS. Then 30ul of 5mg/ml MTT solution was added to each well for 2 hours at 37°C, 5% CO_2. After that, the MTT solution was discarded and 100ul of DMSO was added to each well in order to dissolve the crystal formed by the cells. These plates were incubated for 15 min at 37°C, 5% CO_2. Finally, the plates were measured by ELISA microplate reader (BIO-RAD) at wavelength 540nm.

The results were presented as mean of percentage of survival ± S.D. for the indicated number of different experiments. The percentage of survival cells was calculated by dividing the difference of the absorbance of treated cells and the absorbance of untreated cell multiplied by 100.

2.2.1.4.2.Combined treatment of *Fructus Crataegus* (FC) with LDL-Dox

The relationship between cells and comined treatment of *Fructus Crataegus* (FC) with LDL-Dox could be determined by MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Firstly, cells at 5 x 10^3 cells/well were seeded into the 96-well plate overnight, then the medium was replaced with 1mg/ml of FC in RPMI 1640 with 10% lipoprotein deficient serum (LPDS). The plate was then incubated at 37°C, 5% CO_2 for 48 hours. After 48 hours pre-treatment, the medium was discarded and different concentrations of LDL-Dox in RPMI 1640 with 10% LPDS were added and incubated at 37°C, 5% CO_2 for further 24 hours.

After the treatment, the medium in each well was removed and the cells were washed twice with PBS. Then 30ul of 5mg/ml MTT solution was added to each well for 2 hours at 37°C, 5% CO_2. After that, the MTT solution was discarded and 100ul of DMSO was added to each well in order to dissolve the crystal formed by the cells. These plates were incubated for 15 min at 37°C, 5% CO_2. Finally, the plates were measured by ELISA microplate reader (BIO-RAD) at wavelength 540nm.

The results were presented as mean of percentage of survival ± S.D. for the indicated number of different experiments. The percentage of survival cells was calculated by dividing the difference of the absorbance of treated cells and the absorbance of untreated cell multiplied by 100.

2.2.1.5. The preparation of *Fructus Crataegus* (FC)

The *Fructus Crataegus* extract was passed through S_3y_{10} cartridge dialysis tube whose cut off size was 3000 Dalton. Then the extract would be divided into three parts, the first part was retainable part. This part would be lyophilized and got a powder call "S_3y_{10} Powder". The second part was the permeated powder which would be discarded. The last part was the crude extract which was used for this investigation. Firstly, 0.1N NaOH was used to wash the cartridge materials at pH 13. Then the supernatant was adjust at pH 1.38 followed by centrifuged at 2200x g for 30 min. After that the supernatant was removed, the participate was redissolved by

double distilled water. Then the solution would be lyophilized to get a powder called "0.1N NaOH wash materials".

2.2.1.6. Western blot

The expression level of LDL-R and P-glycoprotein were investigated. Cell at 1 x 10^6 cells/well were seeded in 6-well plate overnight. Then the cells were incubated with different conditions: complete RPMI 1640 medium (CTL), RPMI 1640 medium in 10% lipoprotein deficient serum (LPDS), 1mg/ml FC in 10% LPDS RPMI 1640 medium, 1mg/ml FC and 0.2mg/ml LDL in 10% LPDS RPMI 1640 medium, and 0.2mg/ml LDL in 10% LPDS RPMI medium for 48 hours incubation. After treatment, the medium was removed and the cells were washed twice with 1X PBS and then incubated with trypsin-EDTA. Then the cells were transferred to 1.5ml microfuge tubes followed by centrifuged at 800x g for 5 min. The supernatant was then removed and the cells were washed twice with 1X PBS. The cells were lysed with 100µl lysis buffer which contained 2% SDS, 10% glycerol, 0.0625M Tris-HCl pH 6.8, β-mercaptoethanol (5%v/v), 0.002% bromophenol blue and incubate in ice bath for 30 min. After that, the protein samples were boiled in water bath for 15 min and the samples were stored at -20°C until the time of analysis.

The protein sample was quantified by BCA protein assay. The working BCA protein assay reagent was prepared by combining reagent A and reagent B in the ratio of 50:1. (Reagent A was purchased from Sigma Chemical Company and reagent B was prepared as 4% $CuSO_4 \bullet 5H_2O$). One µl of the samples

mixed up with 19μl of PBS were added to each well of 96-well plate then followed by adding 0.2ml working BCA protein assay reagent. After the plate was incubated at 37°C for 30 min, the absorbance was measured by microplate reader at wavelength 540nm. The concentration of protein could be calculated from the standard curve which was plotted by the absorbance at 540nm against protein content of 0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0μg respectively.

For the Western blot analysis, electrophoresis was performed according to the procedure of Laemmil and Favre (1973) with a Mini-Protean set II apparatus (Bio-Rad), using a 8% resolving gel and a 4.5% stacking gel. Twenty-five μg samples were diluted with the sample loading buffer (10% glycerol, 0.4% SDS, 0.05% bromophenol blue and 20mM EDTA in 0.5M Tris-Cl, pH7.5) followed by the addition of β-mercaptoethanol (5%v/v). The samples were boiled in a water bath for 15 min before loading. Electrophoresis was performed at a constant current of 20mA at room temperature for each gel. After the tracking dye, bromophenol blue, reached the bottom of the gel, electrophoresis was stopped.

Then the gel was transferred to Immobilon-P membrane (Purchased from Millipore Company), treated with 100% methanol and electroblot transfer buffer for 5 min, by semi-dry blotter for 60min at 15mA. After the membranes were incubated in 10% non-fat dry milk at 4°C overnight, they were rinsed three times with TBS-T (1X TBS and 0.1% Tween 20) and rotated for 15 min for three times. Then the membranes were incubated with primary antibody (P-glycoprotein or LDL-R) for 1 hour at room temperature and followed by the secondary antibody (horse-

radish peroxidase linked anti-rabbit antibody for P-glycoprotein and horse-radish peroxidase linked anti-mouse antibody for LDL-R) for 1 hour at room temperature. Finally, all proteins bound to primary antibodies followed by secondary antibodies were detected by ECL Western blotting detection reagents, which were purchased from Amersham Pharmacia Biotech.

2.2.1.7. Flow cytometry

Introduction

Flow cytometry (FCM) has been used extensively to analyze various biological properties of cells. Flow cytometry was a measurement of cells in a flow system that has been designed to deliver particles in single file past a point of measurement. A basic flow cytometry consists of a source of light such as laser beam, a low cell, optical components to focus light of different colors on to the detectors, electronics to amplify and process the resulting signals and a computer (Fig. 2.1). The flow cell is to deliver cells singly to a specific point by hydrodynamic focusing at which the source of light is focused. This is achieved by injection of the sample into the center of a stream of liquid called the sheath fluid. Light source strikes on the cells and the emission of light is collected by detectors. Image analysis by computer was then made to study the distribution of light signals emitting from a population of cells.

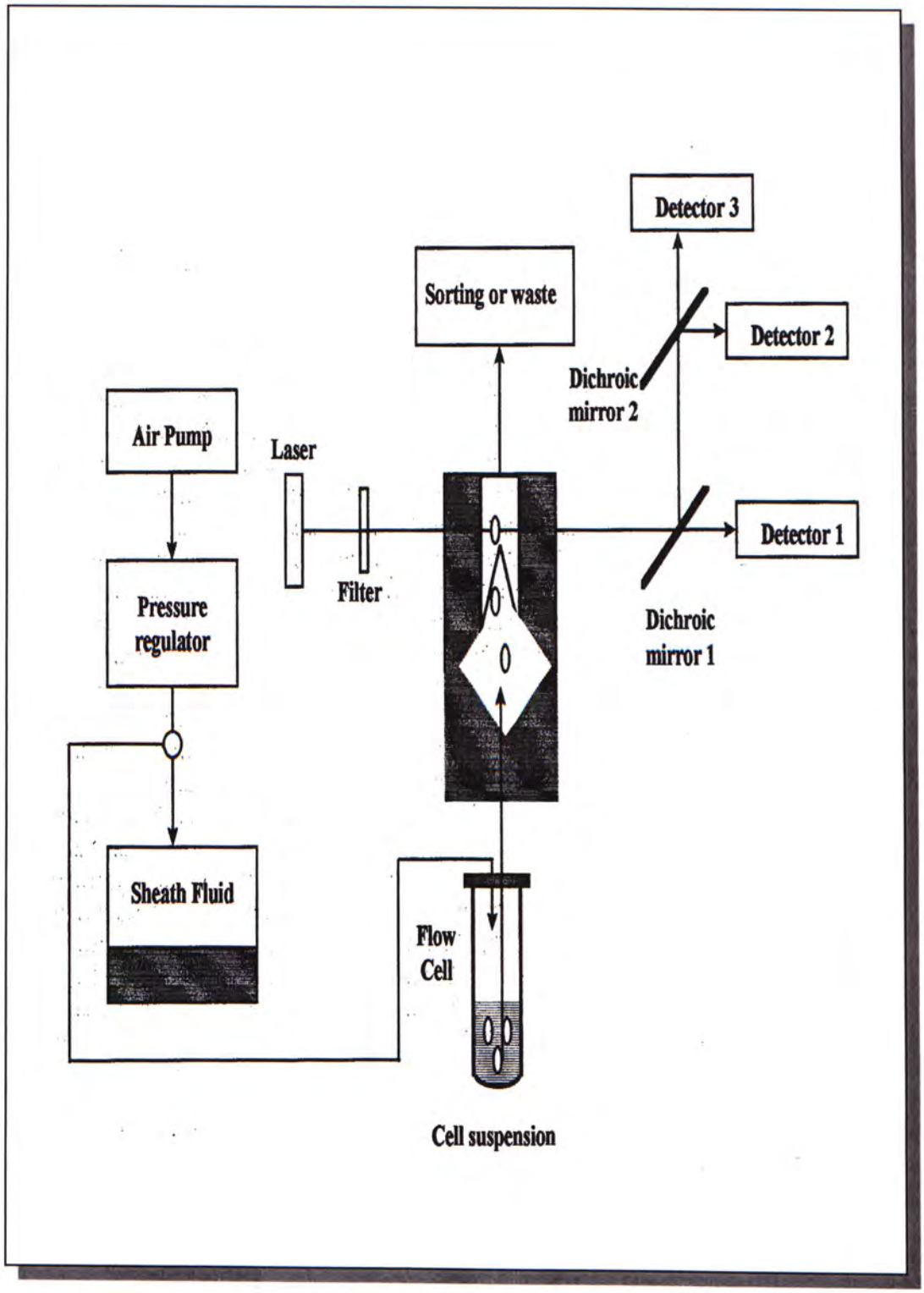


Fig. 2.1. Principle of flow cytometry (Becton Dickinson, FacSort model).

2.2.1.7.1. Procedures for detecting doxorubicin (Dox) and low density lipoprotein-doxorubicin (LDL-Dox) uptake by FCM

Cells at 1 x 10^6 cells/well were seeded in a 6-well plate and incubated at 37°C, 5% CO_2 overnight. The cells were washed by 1X PBS. Then the cells were incubated with different conditions: complete RPMI 1640 medium (CTL), RPMI 1640 medium in 10% lipoprotein deficient serum (LPDS), 1mg/ml FC in 10% LPDS RPMI 1640 medium, 1mg/ml FC and 0.2mg/ml LDL in 10% LPDS RPMI 1640 medium, and 0.2mg/ml LDL in 10% LPDS RPMI medium for 48 hours incubation.

After that, the medium was removed and washed twice by 1X PBS. Then 2ml of 1μM of LDL-Dox in LPDS medium or 1μM of Dox in complete medium was added in the cells for 1 hour incubated at 37°C or 43°C. After the incubation, the treated cells were washed twice with 1X PBS. The adhered cells were trypsinised and centrifuged at about 300x g for 3 minutes. The supernatant was removed and the pellet was then resuspend by RPMI 1640 without phenol red. Finally, the cells were transferred to the flow cytometric tubes. Cells were acquired on the system of FACsort flow cytometer (Becton Dickinson). The acquisition of cells was analysed by Lysys II program (Becton Dickinson). An excitation filter with 488nm wavelength (Argon laser) was selected. The population of cells was determined by forward scatter (FSC) light and side scatter (SSC) light. FSC and SSC determined the size and the granularity of a cell respectively. The signal of doxorubicin was collected at channel FL-2 height (red fluorescence). For the flow cytometric analysis, the fluorescence properties of about 1 x 10^4 cells were collected.

2.2.1.8. Confocal laser scanning microscopy

Introduction

Confocal laser scaning microscopy (CLSM) allows 3-D measurements of biological structures with high spatial contrast. CLSM achieves high resolution of a selected plane in a specimen by means of three basic steps. Light is focused by an objective lens into an hourglass-shaped beam so that the beam strikes one spot at some chosen depth in a specimen (Fig. 2.2). Light reflected from that spot is focused to a front of a detecting device such as photomultiplilier tube (PMT). Meanwhile the opaque regions around the pinhole block out most of the rays that would tend to obscure the resulting image, those reflected by illuminated parts of the specimen lying above and below the plane of interest. Finally, the light is moved rapidly from point to point in the specimen until the entire plane has been scanned.

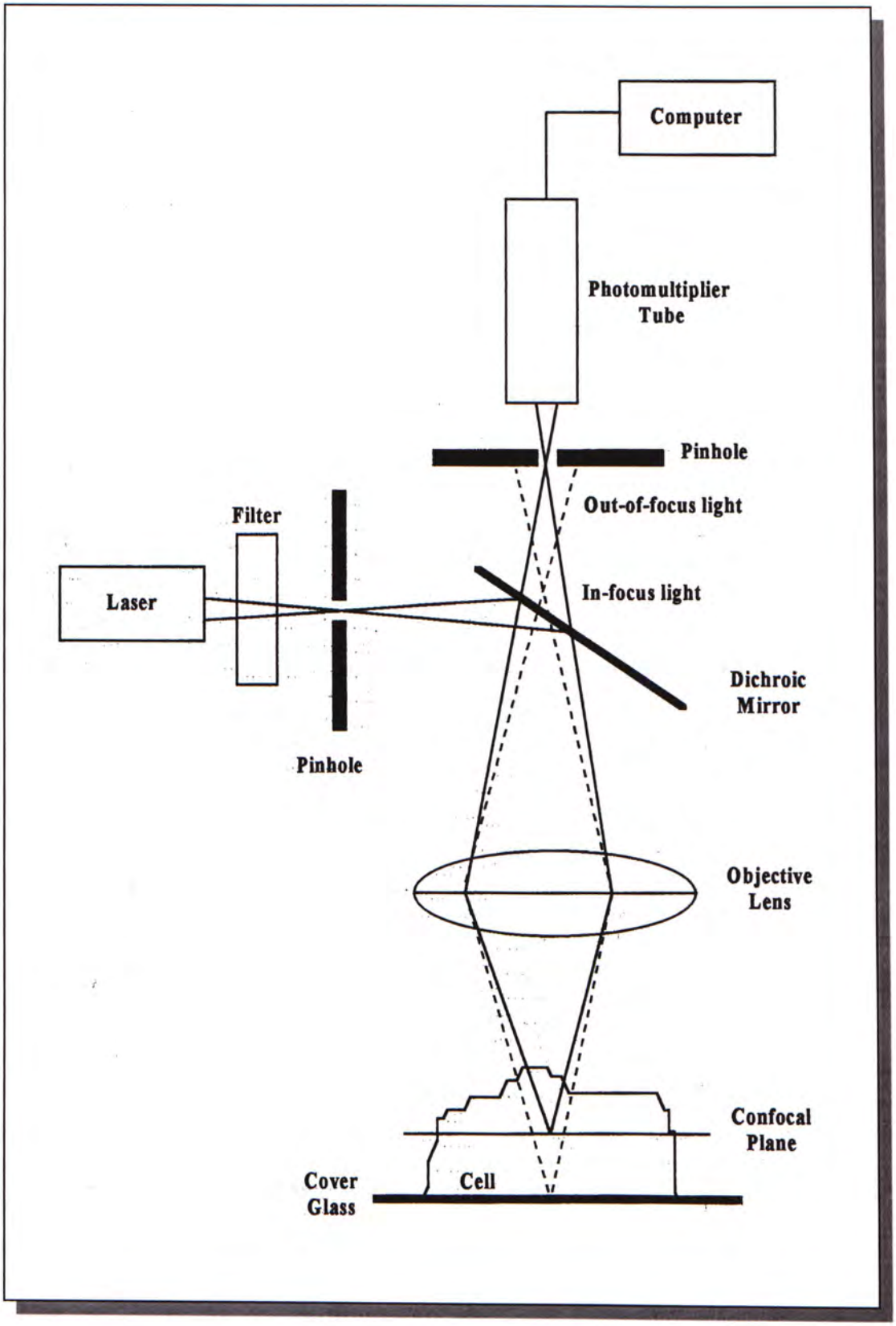


Fig. 2.2. Principle of Confocal laser scaning microscopy.

2.2.1.8.1. Procedures for detecting the cellular of doxorubicin and LDL-DOX

Cells at 1 x 10^4 cells/ml and 2 x 10^4 cells/ml in 1.5ml were seeded on a round cover glass with complete RPMI 1640 medium overnight for HepG2 cells and R-HepG2 cells respectively. The cells were washed twice by 1X PBS. Then the cells were incubated with different conditions: complete RPMI 1640 medium (CTL), RPMI 1640 medium in 10% lipoprotein deficient serum (LPDS), 1mg/ml FC in 10% LPDS RPMI 1640 medium, 1mg/ml FC and 0.2mg/ml LDL in 10% LPDS RPMI 1640 medium, and 0.2mg/ml LDL in 10% LPDS RPMI medium for 48 hours incubation.

After that, the medium was removed and washed by 1X PBS. Then 1.5ml of 5μM of LDL-Dox in LPDS medium or 5μM of Dox in complete medium was added into cells for 1 hour incubation at 37°C or 43°C. After the incubation, the treated cells were washed with RPMI 1640 medium without phenol red twice.

The cover glasses were mounted on a homemade holder followed by adding 0.5ml RPMI1640 medium without phenol red. The cells were then observed under CLSM at room temperature. Images of cells were acquired on Multiprobe 2001 from Molecular Dynamics that fitted with an argon laser 6 mW at excitation and Nikon diaphot inverted microscope. An excitation filter with 488nm wavelength and a long-pass emission filter of 510 nm were used. Cells were scanned by using a 60X (Nikon planApo) or 100X (Nikon, Fluor) oil objectives with low-fluorescence

immersion oil (Stephens Scientifies, USA). The voltage of the Photomultiplier Tube (PMT) was set at optimum. Images were processed by an image analysis software

2.2.2. *In vivo* studies

2.2.2.1. Subcutaneous injection of R-HepG2 cells in nude mouse

Suspension of 8 x 10^6 of human resistant hepatoma R-HepG2 cells were injected subcutaneously (s.c.) into the anterior part of the athymic nude mice.

2.2.2.2. Treatment schedules

Treatments were started 2 days after R-HepG2 cells were implanted onto the mice. The mice were randomly assigned to therapy and control groups of 5 mice a group. The free Dox at dose level of 1mg/kg and 2mg/kg as well as LDL-Dox at dose levels of 1mg/kg were injected into the tail vein on one injection per two days schedule. The injections were given for 4 weeks. Tumor volume was monitored by Vernier caliper measurements weekly and tumor volumes were calculated according to the formula:

Tumor volume (mm^3) =1/2L x 1/2W x H x π

where π = 3.1416

L = Length of the tumor

W = Width of the tumor

H = Height of the tumor

2.2.2.3. Assay of investigating of the myocardial injury

Myocardial injury was accessed by measuring the extent of lactate dehydrogenase (LDH) and creatine kinase (CK) leakage after prolonged treatment of drugs on nude mice. Nude mice bearing R-HepG2 cells were anesthetized with diethyl ether. A 25G syringe, which was pre-washed with Heparin solution, was injected into the heart directly. About 0.8ml per mouse blood was collected in a 1.5ml microtube. Then it was centrifuged at 15,000x g for 15 minutes to separate the red blood cells and plasma. After centrifugation, plasma which at the upper layer was pipetted into a new tube. Plasma LDH activity of coronary effluent was assayed by adding 50ul of plasma into the mixture of 7mM nicotinamide adenine dinucleotide (NADH) and 50mM pyruvate. The reaction mixture in a final volume of 1ml was estimated by monitoring spectrophotomertrically at wavelength 340nm at 30°C. Plasma CK activity of coronary effluent was assayed by adding 20ul of plasma into the mixture of 30mM creatine phosphate, 2mM adenosine diphosphate (ADP), 5mM adenosine monophosphate, 5mM nicotinamide adenine dinucleotide (NAD), 20mM N-acetyl-$_L$-cysteine, 3000U/L Hexokinase (HK) (yeast), 2000U/L glucose-6-phosphate dehydrogenase (G-6-PDH) (L.m.), 10mM magnesium ions, 20mM D-Glucose, 10μM di(adenosine 5')pentaphosphate and 2mM EDTA at pH 6.7 ± 0.1. The reaction mixture in a final volume of 1ml was estimated by monitoring spectrophotomertrically at wavelength 340nm at 30°C.

2.2.2.4. Tissue preparation procedure for light microscope (LM)

The nude mice were sacrificed by cervical dislocation on the day after the last injection of the drugs. Hearts were removed immediately with care handling. The procedures for tissue preparation for light microscopic study were processed by the following:

Procedure		Reagent	Time
Fixing		10% buffered formalin	At least 2 days
Processing:	Dehydration	50% EtOH	1 hour 30 min
		70% EtOH	1 hour 30 min
		85% EtOH	1 hour 30 min
		95% EtOH	2 hours
		Absolute EtOH	45 min
		Absolute EtOH	45 min
		Absolute EtOH	45 min
	Clearing	Xylene: EtOH (1:1)	30 min
		Xylene	30 min
		Xylene	45 min
	Infiltration	Paraffin wax	1 hour 30 min
		Paraffin wax	1 hour 30 min

Embedding: Tissues were embedded in paraffin wax

Sectioning: Tissues were cut into sections of 4 μm in thickness, adhere on slides and dry on oven overnight

Staining:	Dewax	Xylene	5 min
		Xylene	5 min
	Hydration	Absolute EtOH	1 min
		95% EtOH	1 min
		70% EtOH	1 min
		50% EtOH	1 min
		30% EtOH	1 min
		Running tap water	1 min
	Staining	Harris hematoxylin	7 min
		Running tap water	1 min
		Acid EtOH	~ 1-5 sec
		Running tap water	1 min
		Scott's tap water	1 min
		Running tap water	1 min
		0.5% aqueous Eosin	1-2 min
		Running tap water	1 min
	Dehydration	70% EtOH	1 min
		95% EtOH	1 min
		Absolute EtOH	2 min
		Absolute EtOH	2 min
		Xylene: EtOH (1:1)	2 min
		Xylene	2 min
		Xylene	2 min

Mounting: Slides were mounted with Canada Balsam

The sections were observed under light microscope (OLYMPUS) by different combination of magnification values of the eyepieces and objective lens of the microscope. Pictures of the heart sections under microscope were taken for record and comparison.

2.2.3. Statistical analysis in our research

Statistical analysis was done using the Student's t-test.

CHAPTER 3 : RESULTS

3.1. *IN VITRO* STUDIES

3.1.1. The preparation of low density lipoprotein-doxorubicin (LDL-Dox)

After LDL was mixed up with Dox in shaking air-bath at 37°C for 24 hours in dark, the mixture was then centrifuged at 800x g for 3 minutes and loaded onto G25 Sephadex column using 0.9% saline as running buffer to separate the free Dox from LDL-Dox complex. In each fraction, 0.5ml aliquots were collected.

The collecting fractions were monitored at wavelength 480nm and 280nm to determine the LDL-Dox concentration. The graph Fig. 3.1 showed that there was only one peak at wavelength 280nm which represented the absorbance of protein and there were two peaks at wavelength 480nm which represented the absorbance of Dox. The peak overlapped at wavelength 280nm and 480nm represented the LDL-Dox and the peak only appeared at wavelength 480nm represented the free Dox.

After using Lowery's method to measure the protein contents in the collecting fractions, only fraction 5, 6, and 7 were found to contain protein. These fractions were pooled together to measure the concentration of Dox in LDL-Dox complex. These pooled fractions were dissolved in acidified isopropanol in order to release Dox from LDL.

According to the pilot studies in our laboratory, it showed that the LDL in the pooled fractions did not affect the absorbance of Dox in acidified isopropanol.

The LDL-Dox used in the following investigation was only collected from the first few fractions of the mixture (fraction 5, 6, and 7) to ensure that these fractions only contained LDL-Dox, but not the free Dox which appeared from fractions 11 to 20.

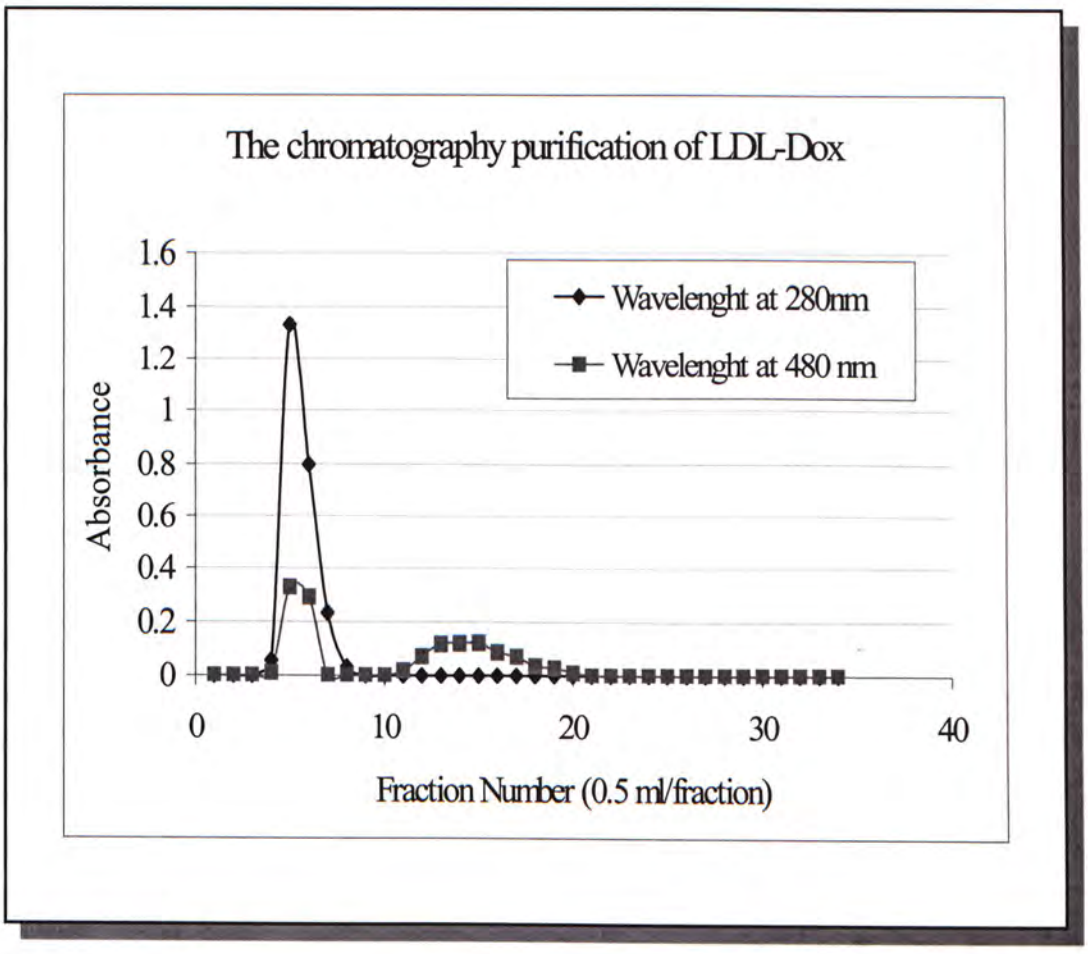
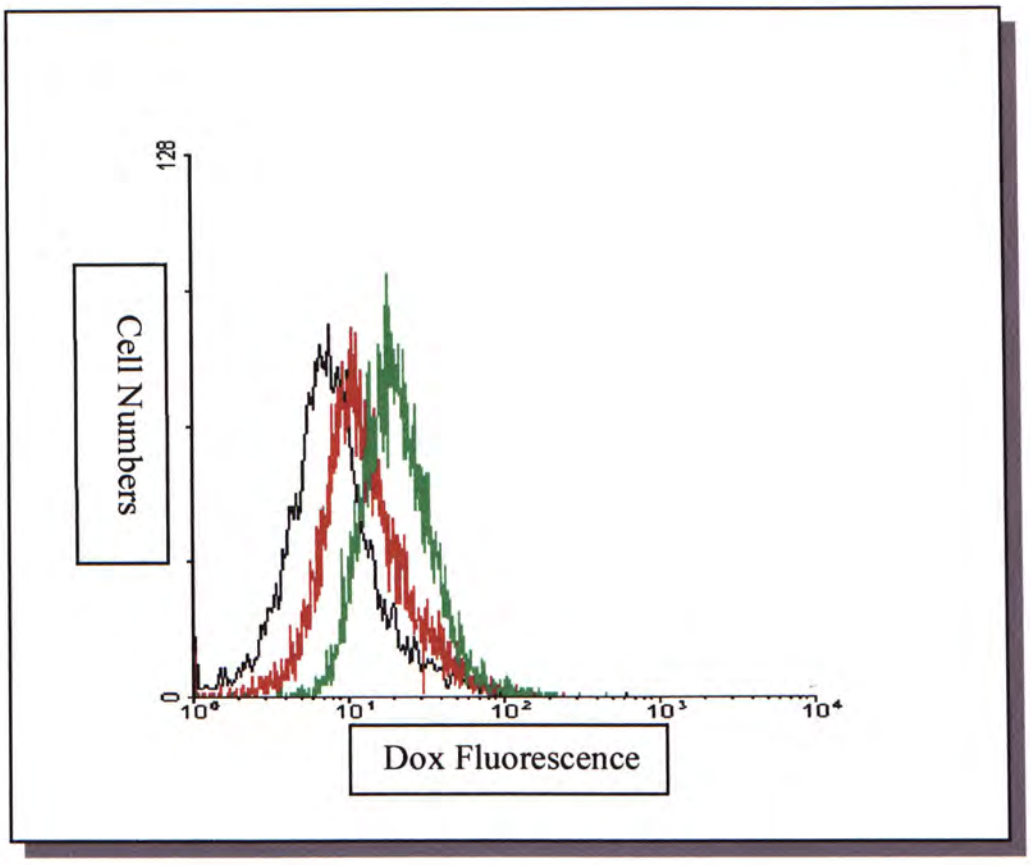


Fig. 3.1. The chromatography of purification of low density lipoprotein-doxorubicin (LDL-Dox). The mixture of 6.2mg low density lipoprotein (LDL) and 0.62mg doxorubicin (Dox) were incubated in 37°C shaking air bath at dark environment for 24 hours. After incubation, the mixture was centrifuged at 800x g for 3 min in a desktop centrifuge. The supernatant was loaded into a 5ml pre-packed G-25 Sephadex column evenly. After the sample just ran into the column, 0.9% of saline was applied as a running buffer. Fractions of 0.5ml were collected in the microtubes. The absorbance at each tube was measured at the wavelength of both 280nm and 480nm.

3.1.2 Studies on human hepatoma cells line (HepG2 cells)

3.1.2.1.The comparison of Dox and LDL-Dox accumulated in HepG2 cells

Since the expression level of low density lipoprotein receptor (LDL-R) on tumor cells was high, it is interested to test whether the accumulation of Dox in LDL-Dox treated cells was higher than that of free Dox treated tumor cells. Fig. 3.2 shows the comparison of Dox and LDL-Dox accumulated in HepG2 cells. After measuring the accumulation of drug in HepG2 cells, it showed that the mean of the fluorescent intensity of cells on Dox treatment was 11.76 and the mean of the fluorescent intensity of cells on LDL-Dox treatment was 19.46. It indicated that the accumulation of Dox in LDL-Dox treated cells was higher than that of free Dox treated cells.



Sample	Mean Fluorescence Value / units
Control	7.10
Dox	11.76
LDL-Dox	19.46

Fig. 3.2. The quantitative analysis of doxorubicin and low density lipoprotein-doxorubicin accumulated in HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6-well plate for 48 hours. Cells were treated with 1μM Dox in complete medium or LDL-Dox in lipoprotein deficient serum for 1 hour. The accumulation of Dox and LDL-Dox was measured by flow cytometric analysis. The black line represented the control cells, i.e. cells incubated without Dox or LDL-Dox. The red line represented the cells incubated with 1μM Dox. The green line represented the cells incubated with 1μM LDL-Dox.

3.1.2.2. Confocal laser scanning microscopic (CLSM) studies on the accumulation of Dox and LDL-Dox in HepG2 cells

According to the result of flow cytometric analysis, it showed that the level of LDL-Dox accumulated in the HepG2 cells was more than that of Dox. Since Dox had fluorescent, the comparison of Dox and LDL-Dox accumulated in the cells can also be monitored by the confocal laser scanning microscopy (CLSM). The higher fluorescent intensity, the more Dox or LDL-Dox was present in the cells. In Fig. 3.3, the results of confocal laser scanning microscopic images of Dox-treated cells and LDL-Dox-treated cells were shown in pseudo-color. The warm color represented a high degree of fluorescence whereas cool color represented a low degree of Dox fluorescence.

From the graph, it found that the cells treated with LDL-Dox showed a higher degree of fluorescent intensity than that treated with Dox. This result suggested that the intracellular level of LDL-Dox was higher than that of Dox which confirmed the result that more accmulation of LDL-Dox than Dox were found in the cells.

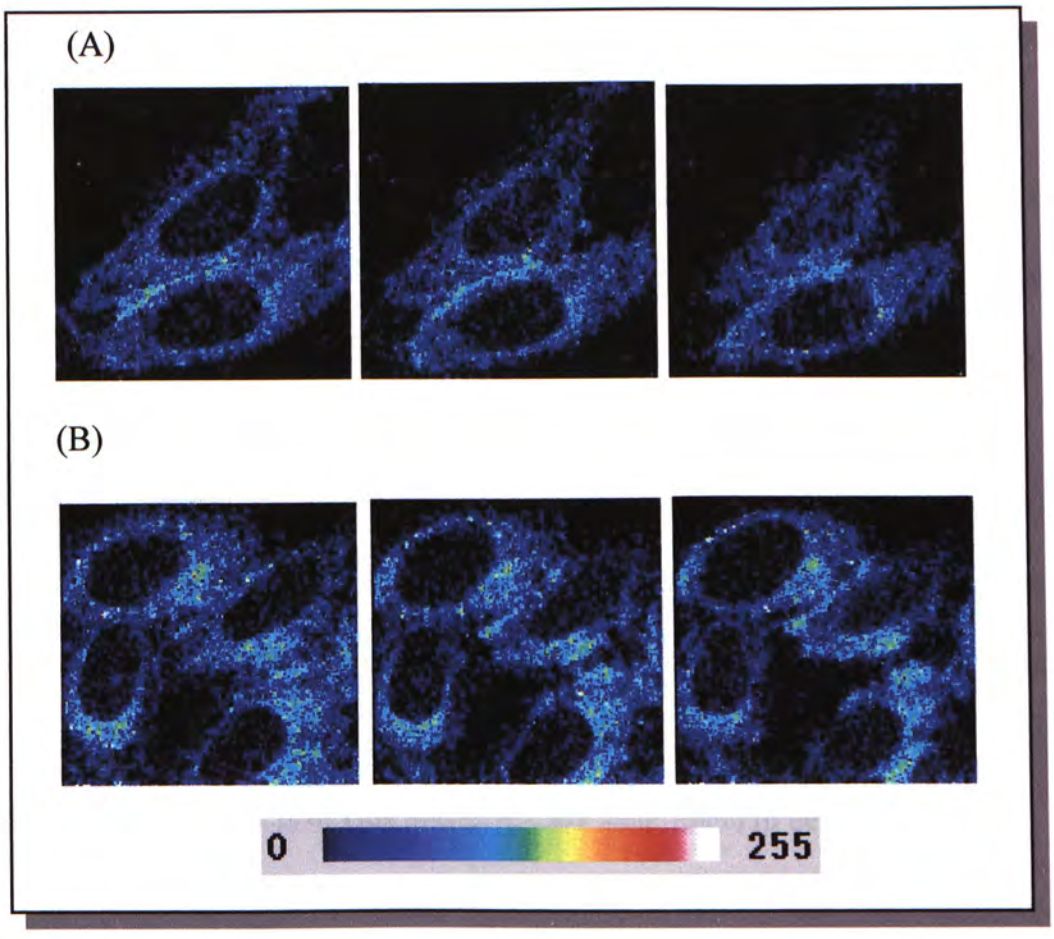


Fig. 3.3. The confocal laser scanning microscopic analysis on the accumulated level of Dox and LDL-Dox in HepG2 cells. Cells at 5 x 10^4 were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM Dox or LDL-Dox at 37°C 5% CO_2 for 1 hour. (A), Dox-treated HepG2 cells were scanned along the Z-axis for 1μm. (B), LDL-Dox-treated HepG2 cells were scanned along Z-axis for 1μm.

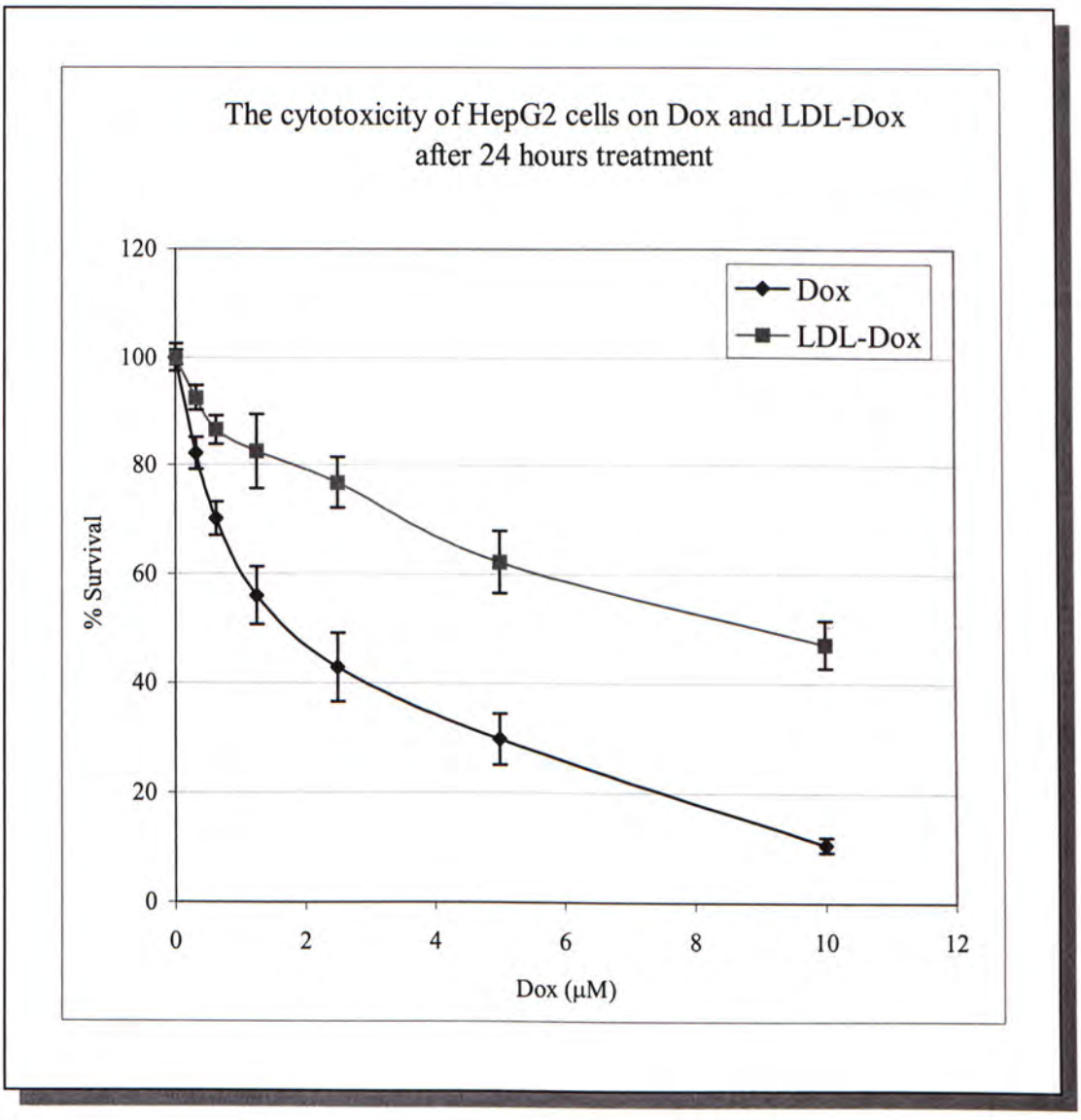
3.1.2.3.The comparsion of the cytotoxicity of Dox and LDL-Dox on HepG2 cells

The HepG2 cells were incubated at different concentrations of Dox and LDL-Dox ranging from 10μM to 0.3125μM under 37°C, 5% CO_2 for 24 hours, 48 hours and 72 hours respectively. The cell viability was determined by MTT assay.

When the cells were incubated for 24 hours, the IC_{50} of Dox and LDL-Dox were found to be 1.7μM and 8.9μM respectively (Fig. 3.4). In addition, when the incubation time was prolonged from 24 hours to 48 hours, the IC_{50} of Dox and LDL-Dox were found to be 0.6μM and 4.7μM respectively (Fig. 3.5). After 72 hours incubation, the IC_{50} of Dox and LDL-Dox were found to be 0.3μM and 3.6μM respectively (Fig. 3.6).

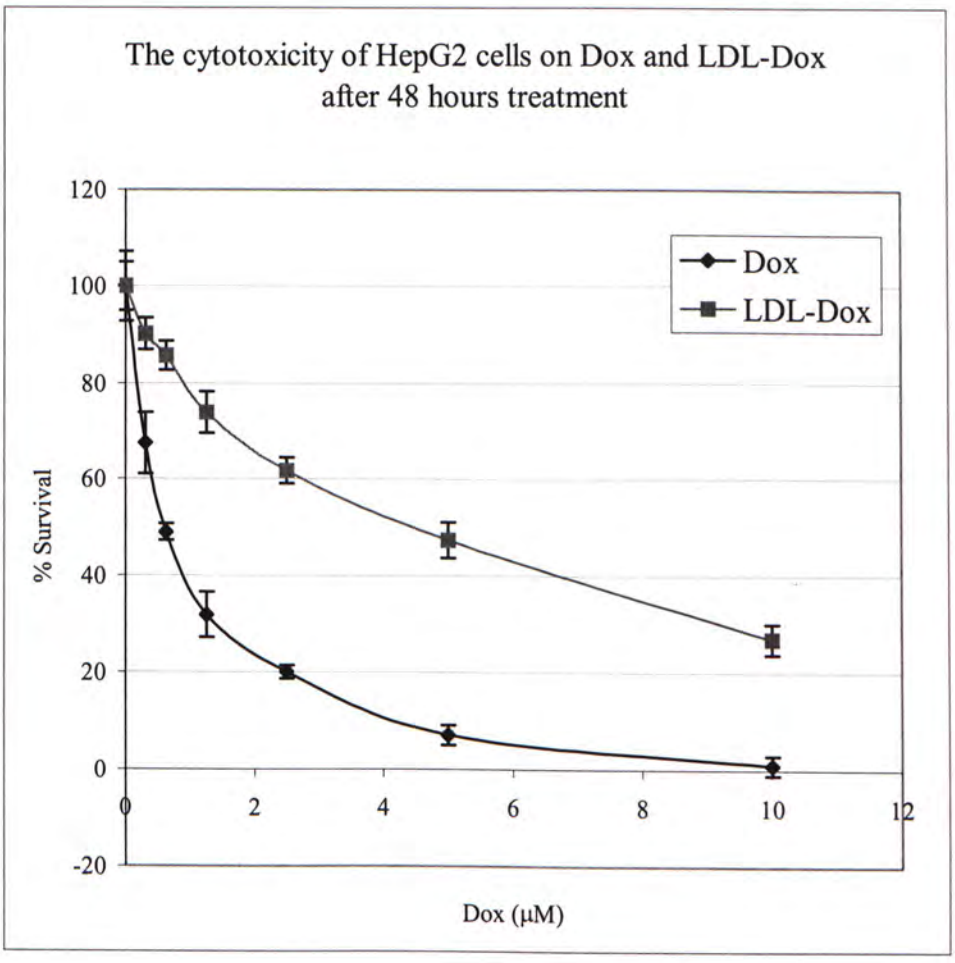
These results showed that as the concentrations of Dox and LDL-Dox was increased, the values of cell viability were decreased, i.e. the percentage of cell survival was dose-dependent (Fig. 3.7). When the incubation time was prolonged, the values of cell viability were also decreased, i.e. the percentage of cells survival was time-dependent (Fig. 3.8). Hence, the percentage survival of HepG2 cells was time- and dose- dependent on incubation of Dox and LDL-Dox.

On the other hand, the results also indicated that after Dox coupling into the LDL, its cytotoxic effect on HepG2 cells was decreased. This result implied it might need a longer time for Dox to release from LDL-complex.



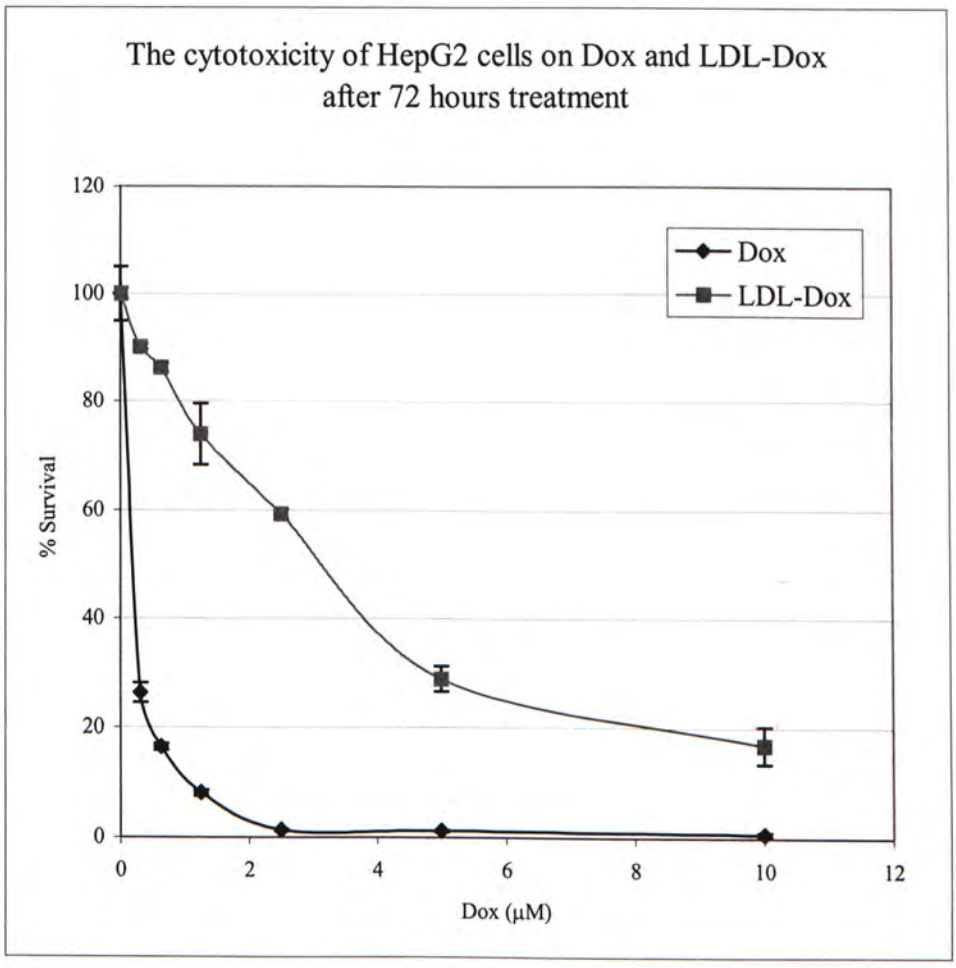
Sample	The value of IC_{50}
Dox	1.7
LDL-Dox	8.9

Fig. 3.4. Effect of doxorubicin and low density lipoprotein-doxorubicin on the survival of HepG2 cells after 24 hours incubation. Cells at 2 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of Dox and LDL-Dox for 24 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



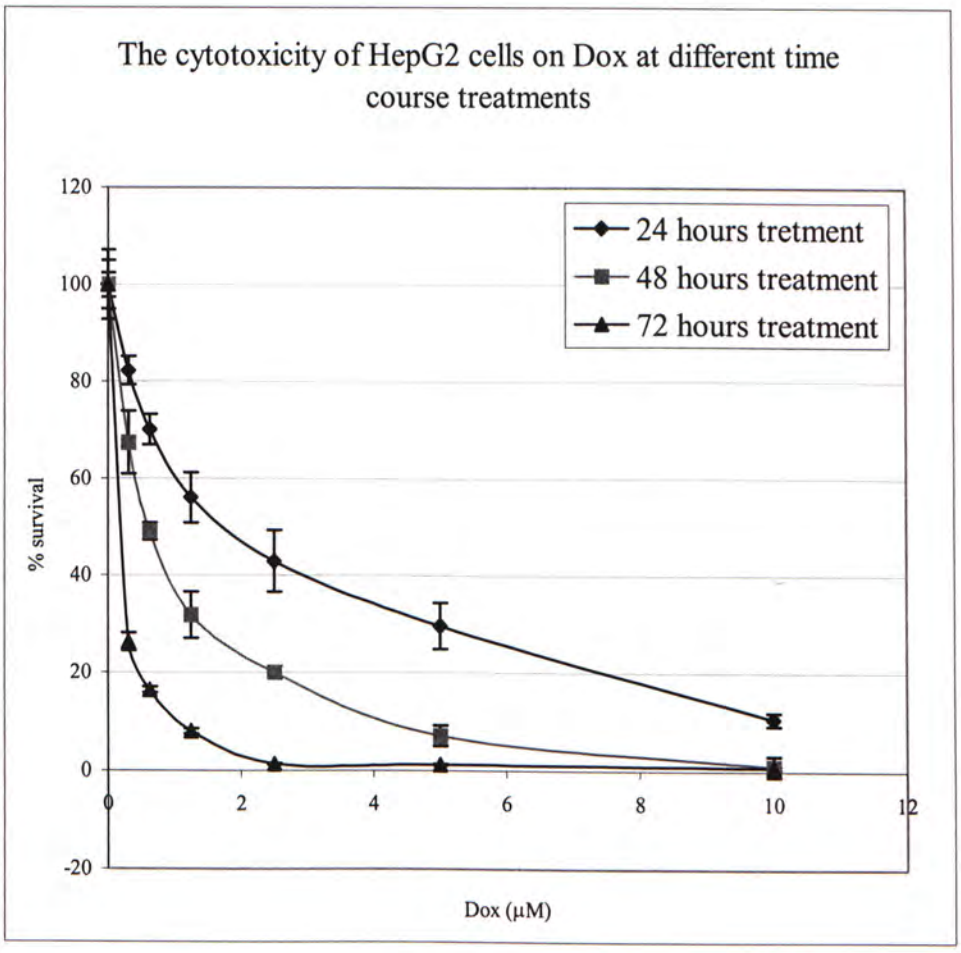
Sample	The value of IC_{50}
Dox	0.6
LDL-Dox	4.7

Fig. 3.5. Effect of doxorubicin and low density lipoprotein-doxorubicin on the survival of HepG2 cells after 48 hours incubation. Cells at 1 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of Dox and LDL-Dox for 48 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



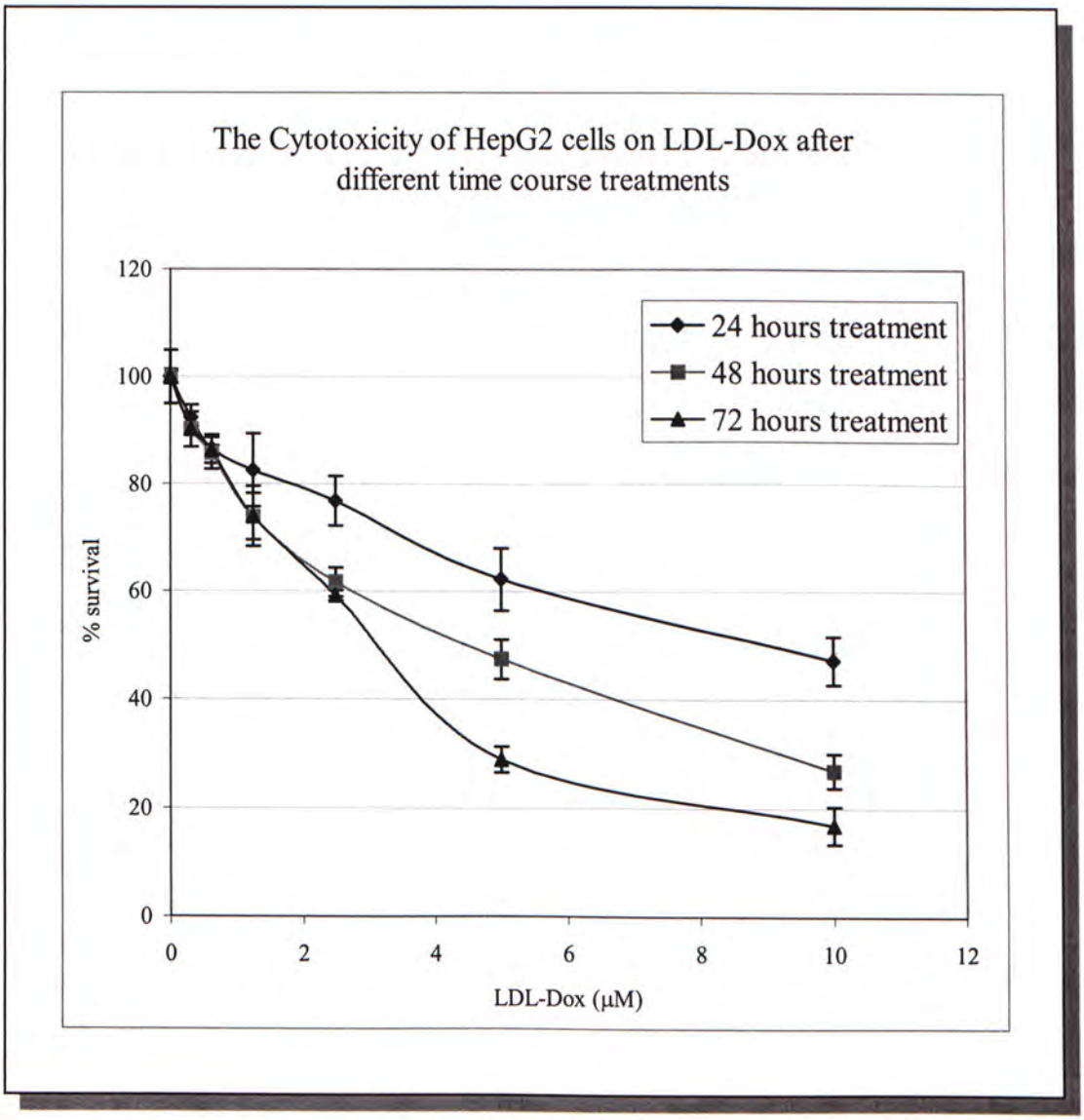
Sample	The value of IC_{50}
Dox	0.3
LDL-Dox	3.6

Fig. 3.6. Effect of doxorubicin and low density lipoprotein- doxorubicin on the survival of HepG2 cells after 72 hours incubation. Cells at 3 x 10^5 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of Dox and LDL-Dox for 72 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



Time course	The value of IC_{50} on Dox
24 hours	1.7
48 hours	0.6
72 hours	0.3

Fig. 3.7. Effect of doxorubicin on the survival of HepG2 cells at different time course treatments. Cells at 2 x 10^4, 1 x 10^4 and 3 x 10^5 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of doxorubicin for 24, 48 and 72 hours respectively. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



Time course	The value of IC_{50} on LDL-Dox
24 hours	8.9
48 hours	4.7
72 hours	3.6

Fig. 3.8. Effect of low density lipoprotein-doxorubicin on the survival of HepG2 cells at different time course treatments. Cells at 2 x 10^4, 1 x 10^4 and 3 x 10^5 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of LDL-Dox for 24, 48 and 72 hours respectively. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.

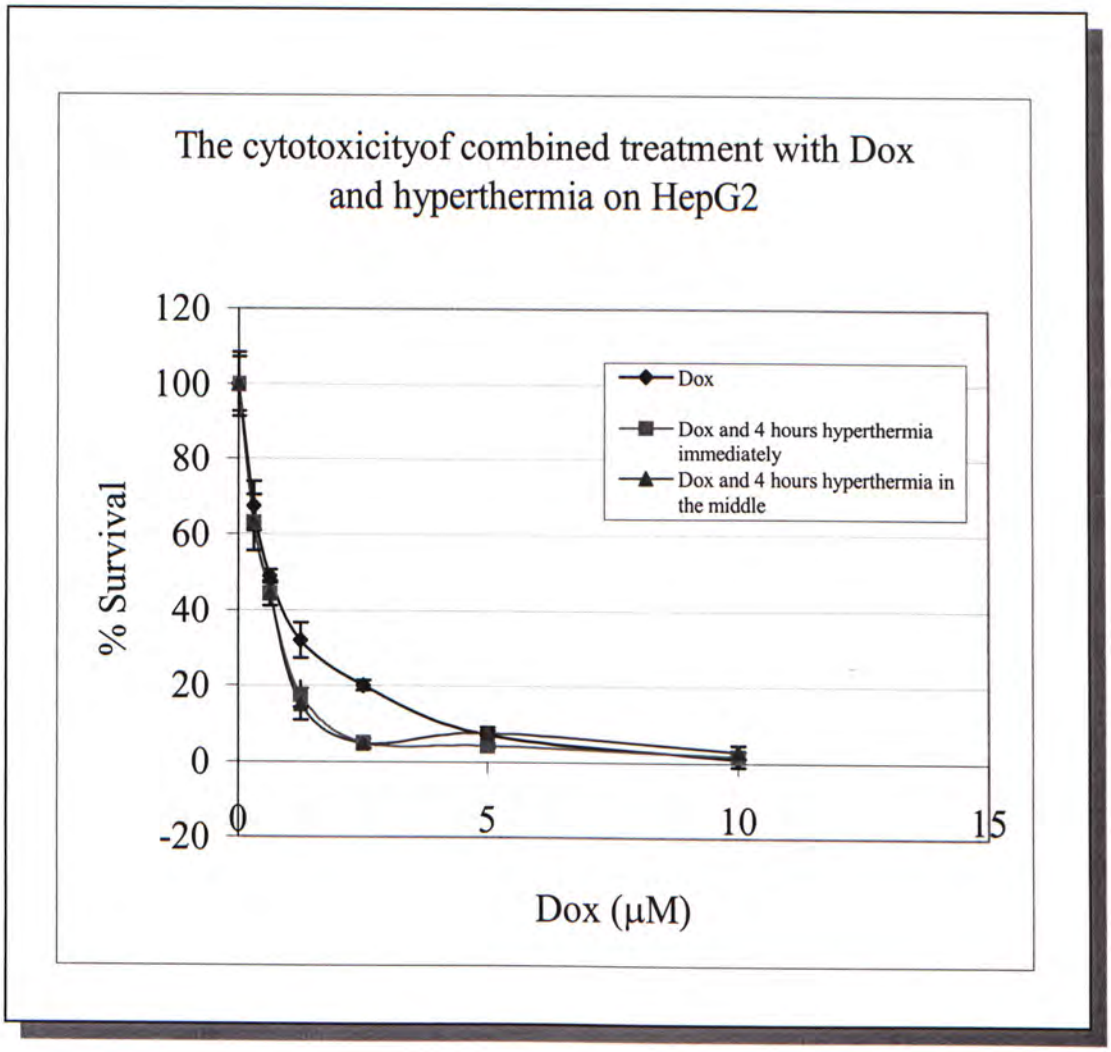
3.1.2.4.The comparison of the cytotoxicty of Dox and LDL-Dox with and without hyperthermia on HepG2 cells

When the HepG2 cells were incubated at 43°C at different time periods, the cytotoxic effect of Dox and LDL-Dox were higher than those obtained at 37°C. HepG2 cells were incubated at 43°C for 4 hours at two different periods, namely hyperthermia was applied at the beginning of drugs treatment, i.e. at 0 hour to 4 hours during the treatment and hyperthermia was applied in the middle of the drug treatment, i.e. at 22 hours to 26 hours during the treatment. For the Dox treatment, the cytotoxicity of Dox under hyperthermia was higher than that without hyperthermia. Moreover, the effect of hyperthermia applied at the beginning or in the middle of the drug treatment was similar (Fig. 3.9).

For LDL-Dox treatment, when the cells were incubated at 43°C, the cytotoxic effect of LDL-Dox either adding LDL-Dox immediately or in the middle of the treatment was also increased when compared with those obtained by LDL-Dox treatments only. That means the cytotoxicity of LDL-Dox combined with hyperthermia was higher than that without hyperthermia (Fig. 3.10). The hyperthermia effect occurred after adding drug immediately was similar to that occurred in the middle of drug treatment.

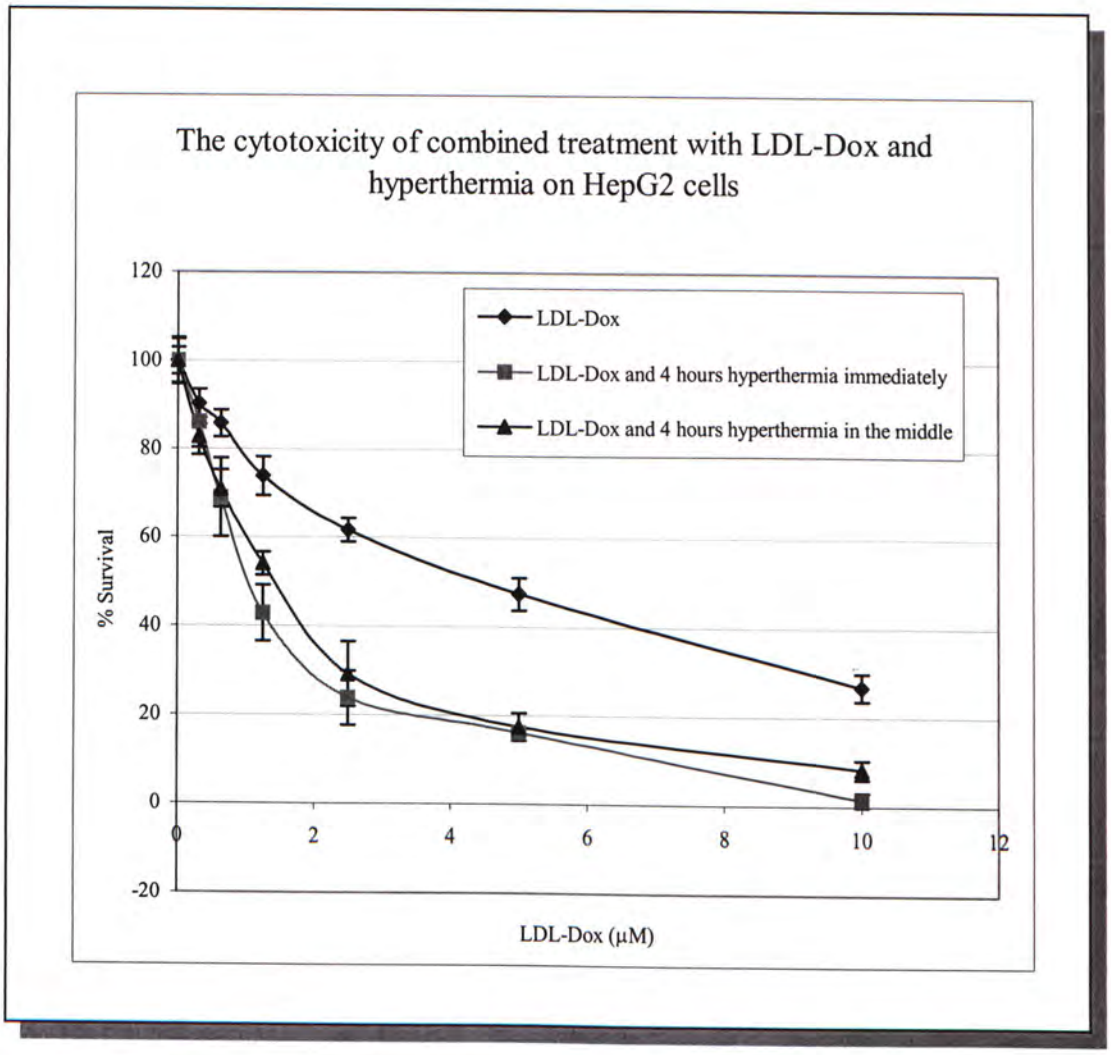
According to the above results, it suggested that the hyperthermia could enhance the cytotoxic effect of Dox and LDL-Dox on HepG2 cells. Furthermore, the hyperthemia effect did not have a significant difference on the Dox

and LDL-Dox treatments no matter whether hyperthermia was applied at the beginning or in the middle of the drug treatment.



Sample	The value of IC_{50}
Dox only	0.6
Dox and hyperthermia immediately	0.3
Dox and hyperthermia in the middle	0.4

Fig. 3.9. Effect of combined treatment with doxorubicin and hyperthermia on the survival of HepG2 cells. Cells at 1 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of Dox for 48 hours. During the incubation time, the hyperthermia treatment was applied immediately after adding drugs or in the middle of the drug treatment. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



Sample	The value of IC_{50}
LDL-Dox only	4.7
LDL-Dox and hyperthermia immediately	2.1
LDL-Dox and hyperthermia in the middle	2.8

Fig. 3.10. Effect of combined treatment with low density lipoprotein-doxorubicin and hyperthermia on the survival of HepG2 cells. Cells at 1 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of LDL-Dox for 48 hours. During the incubation time, the hyperthermia was applied immediately after adding drugs or in the middle of the drug treatment. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.

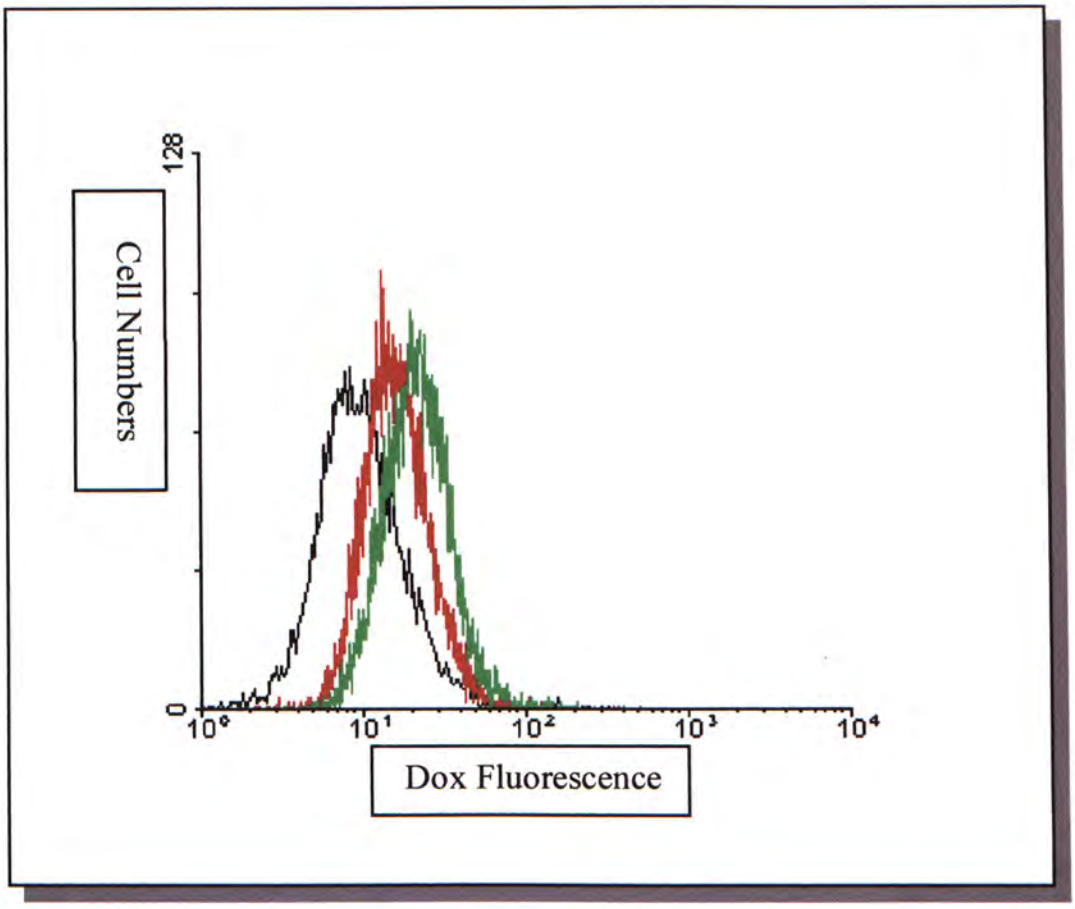
3.1.2.5.The comparison of accumulation of Dox and LDL-Dox in HepG2 cells treated with and without combination of hyperthermia

The results of Dox and LDL-Dox combined with hyperthermia indicated that hyperthermia could enhance the cytotoxic effect of Dox and LDL-Dox. Whether this effect was enhanced by the accumulation of Dox and LDL-Dox in the cells were examined by flow cytometric analysis.

When comparing between the accumulations of 1μM of Dox in the HepG2 cells incubated at 37°C and 43°C both at 5% CO_2 1 hour, the mean of the fluorescent intensity of cells incubated at 37°C was 15.12 and the mean of the cells incubated at 43°C was 21.88. There was an increase in the accumulation of Dox into the cells incubated at 43°C when compared with those incubated at 37°C, which implied that increased intracellular accumulation of Dox from 37°C to 43°C could be observed (Fig. 3.11).

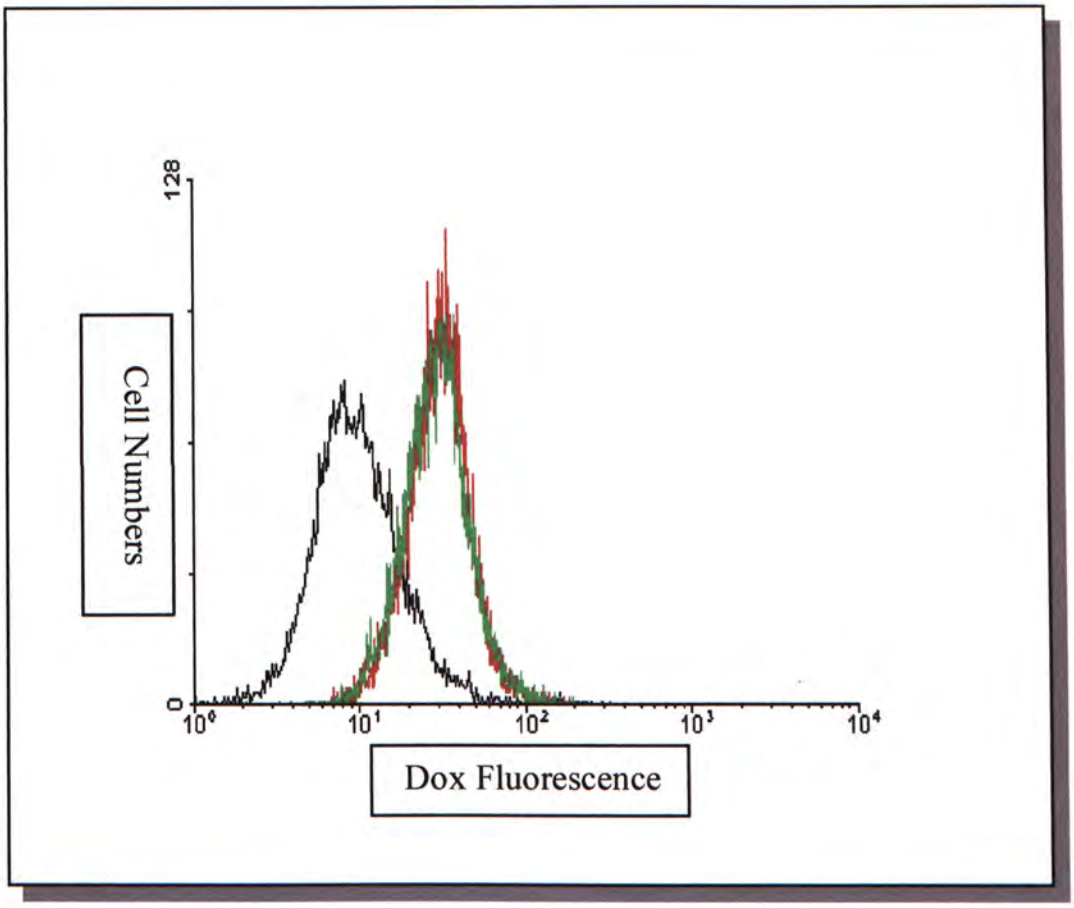
In addition, when comparing between the accumulation of 1μM of LDL-Dox in the HepG2 cells incubated at 37°C and 43°C both at 5%CO_2 for 1 hour, the mean of the fluorescent intensity of cells incubated at 37°C and 43°C was both 30.23. There was no clear difference between the accumulation of LDL-Dox in the cells under hyperthermia or not (Fig. 3.12).

These results showed that hyperthermia could enhance the accumulation of Dox in the cells, but not for LDL-Dox.



Sample	Mean Fluorescence Value / units
Control at 37°C	9.39
Dox at 37°C	15.12
Dox at 43°C	21.88

Fig. 3.11. The quantitative analysis of doxorubicin accumulated in HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6-well plate for 48 hours. Cells were treated with 1μM Dox for 1 hour at 37°C or 43°C. The accumulation of Dox was measured by flow cytometric analysis. The black line represented the control cells, i.e. cells incubated without Dox at 37°C. The red line represented the cells incubated with 1μM Dox at 37°C. The green line represented the cells incubated with 1μM LDL-Dox at 43°C.



Sample	Mean Fluorescence Value / units
Control at 37°C	9.39
LDL-Dox at 37°C	30.23
LDL-Dox at 43°C	30.23

Fig. 3.12. The quantitative analysis of low density lipoprotein-doxorubicin accumulated in HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6-well plate for 48 hours. Cells were treated with 1μM LDL-Dox for 1 hour at 37°C or 43°C. The accumulation of LDL-Dox was measured by flow cytometric analysis. The black line represented the control cells, i.e. cells incubated without LDL-Dox at 37°C. The red line represented the cells incubated with 1μM LDL-Dox at 37°C. The green line represented the cells incubated with 1μM LDL-Dox at 43°C.

3.1.2.6.Confocal laser scanning microscopic (CLSM) studies on the accumulation of Dox and LDL-Dox in HepG2 treated cells with and without hyperthermia

The result of flow cytometric analysis showed that after the cells incubated at 43°C with Dox, the accumulation level of Dox in the cells was higher than that of the cells incubated at 37°C with Dox. From the confocal laser scanning microscopic images of Dox-treated cells at 37°C and 43°C, it showed that those observed in 43°C-treated cells showed a higher fluorescent intensity when compared with those observed in 37°C-treated cells. This result suggested that the intracellular level of Dox was enhanced by hyperthermia (Fig. 3.13).

In addition, when comparing the level of LDL-Dox accumulated in the cells incubated between at 37°C and 43°C, the fluorescent intensity of LDL-Dox in cells incubated at 37°C was similar to that observed in cells incubated at 43°C, indicating that there was no clear difference between the effect in cells treated with hyperthermia or not (Fig. 3.14).

These results suggested that the combined treatment of Dox and hyperthermia could enhance the intracellular level of Dox, but hyperthermia could not enhance the intracellular level of LDL-Dox. These results were comparable to those of flow cytometric analyses.

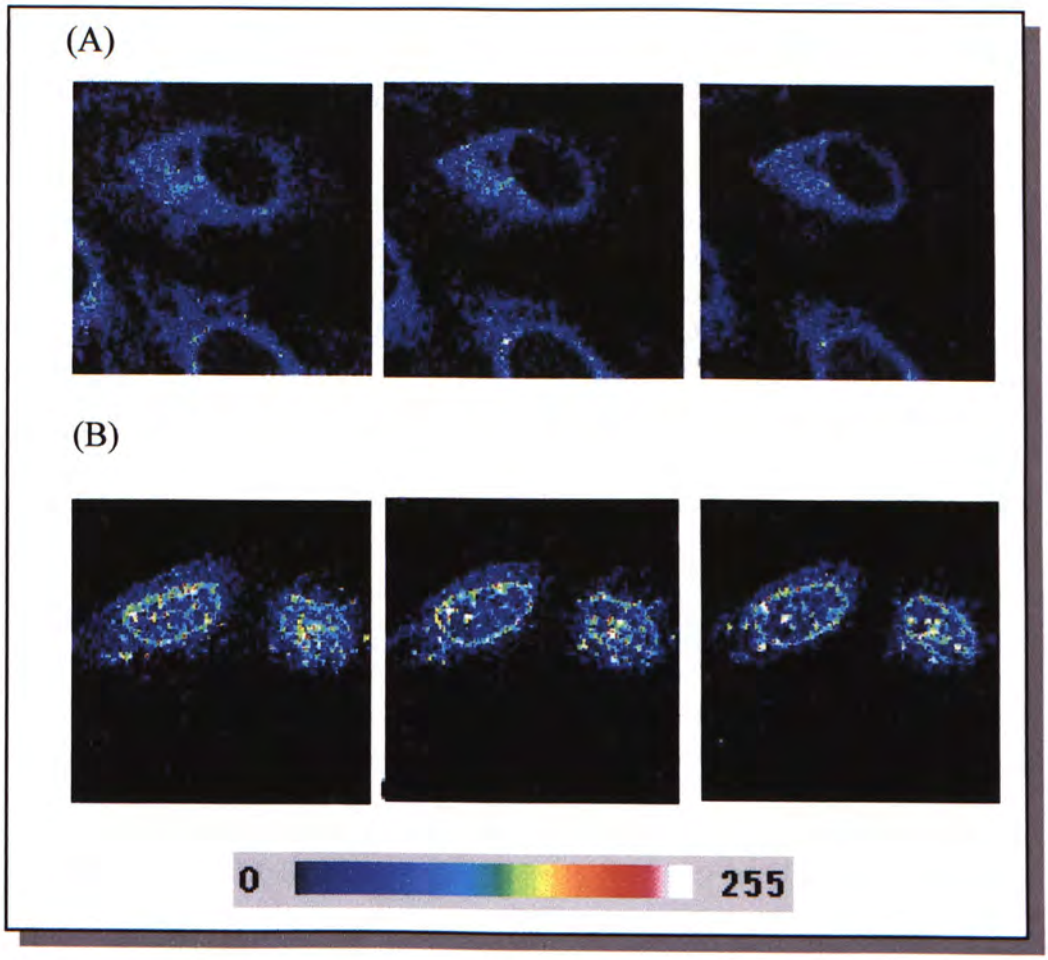


Fig. 3.13. The confocal laser scanning microscopic analysis on the accumulated level of Dox in HepG2 cells. Cells at 5 x 10^4 cells were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM Dox for 1 hour at 37°C or 43°C. (A), Dox-treated HepG2 cells incubated at 37°C were scanned along the Z-axis for 1μm. (B), Dox-treated HepG2 cells incubated at 43°C were scanned along Z-axis for 1μm.

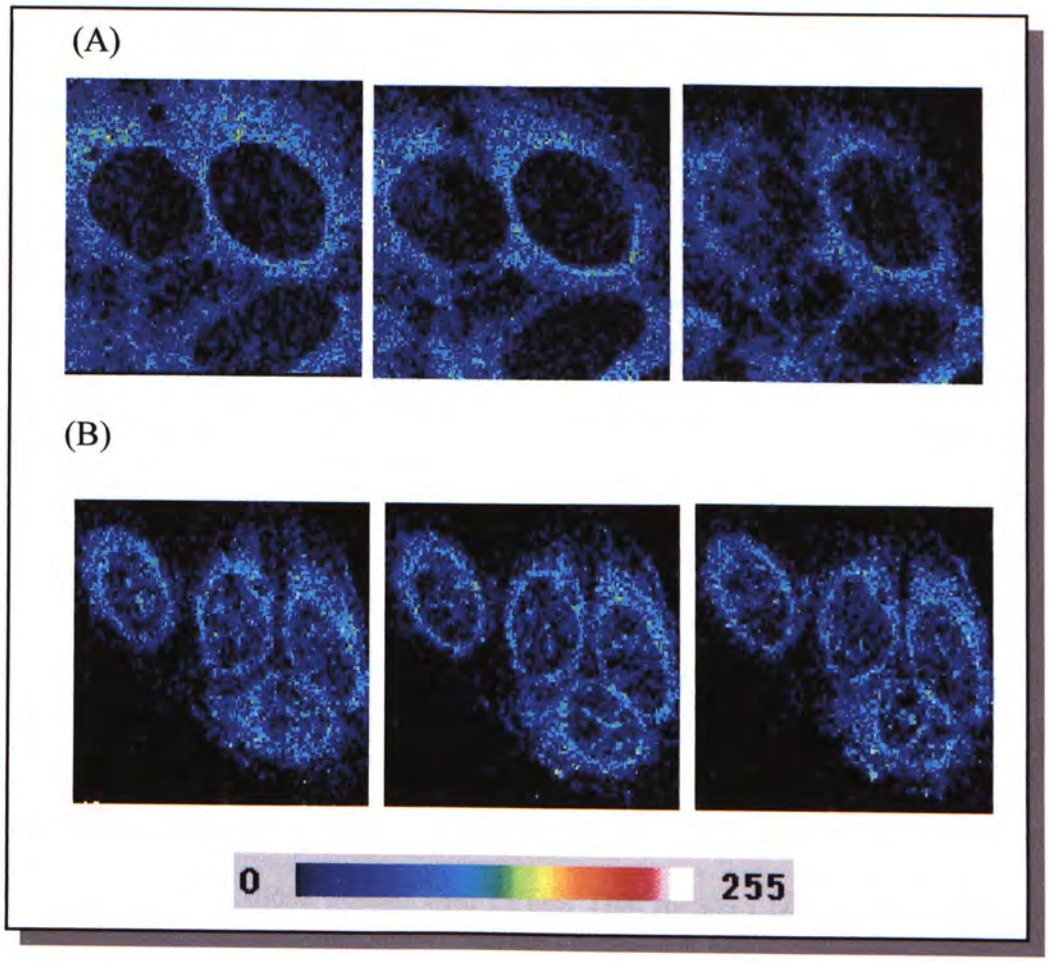


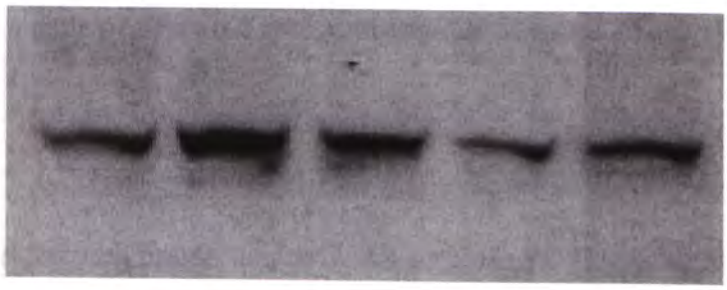
Fig. 3.14. The confocal laser scanning microscopic analysis on the accumulated level of LDL-Dox in HepG2 cells. Cells at 5 x 10^4 cells were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM LDL-Dox for 1 hour at 37°C or 43°C. (A), LDL-Dox-treated HepG2 cells incubated at 37°C were scanned along the Z-axis for 1μm. (B), LDL-Dox-treated HepG2 cells incubated at 43°C were scanned along Z-axis for 1μm.

3.1.2.7.Modulation of LDL receptors on HepG2 cells ------ Up-regulation of LDL receptors by *Fructus Crataegus* (FC)

3.1.2.7.1.The comparsion of LDL receptor expression on HepG2 cells after *Fructus Crataegus* (FC) pre-treatment

Results in Fig. 3.15 demonstrated that in HepG2 cells, the LDL receptor expression levels in various treatments were different. The cells treated with lipoprotein deficient serum (LPDS) showed a little bit higher LDL receptor expression than that with complete medium (control). Moreover, the cells treated with low density lipoprotein (LDL) only exhibited less LDL receptor expression when compared with those treated with LPDS. That means LDL could suppress the LDL receptor expression. After the cells were treated with *Fructus Crataegus* (FC) only, the expression level of LDL receptor was higher than the cells treated with LPDS, i.e. the FC might up-regulate LDL receptor expression. When the cells were co-treated with FC and LDL, the expression level was reduced when compared with that in FC-treated cells, but it was still higher than that in cells treated with LDL only. These results indicated that the expression level of LDL receptor increased by the effect of FC was higher than that suppressed by the effect of LDL.

The result suggested that FC could up-regulate the expression level of LDL receptors and LDL could suppress the expression of LDL receptors on HepG2 cells.



Lane 1: Complete medium (CTL)

2: FC (1mg/ml) in LPDS

3: FC (1mg/ml) and LDL (0.2mg/ml) in LPDS

4: LDL (0.2mg/ml) in LPDS

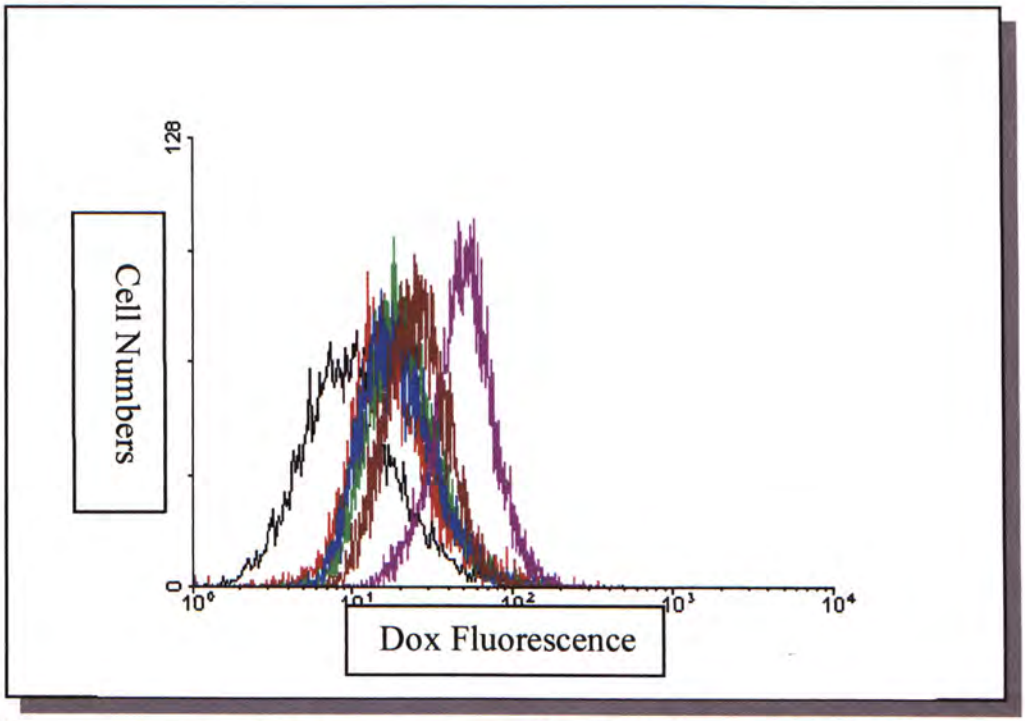
5: Lipoprotein deficient serum (LPDS)

Fig. 3.15. The expression level of LDL receptors in various treatments in HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6 well-plate overnight. Cells were treated with complete medium (CTL), lipoprotein deficient serum (LPDS), *Fructus Crataegus* (FC) in lipoprotein deficient serum, co-treatment of *Fructus Crataegus* and low density lipoprotein (FC and LDL) in lipoprotein deficient serum, and low density lipoprotein (LDL) in lipoprotein deficient serum. The level of LDL receptor was analyzed by Western blot analysis. Aliquots of 25μg of protein was loaded in each lane.

3.1.2.7.2.The comparison of accumulation of LDL-Dox accumulated in HepG2 cells pre-treated with and without *Fructus Crataegus* (FC)

As observed in Fig. 3.16, the accumulation of LDL-Dox in HepG2 cells treated with lipoprotein deficient serum (LPDS) was higher than that when cells were treated with complete medium (control). The mean of the fluorescent intensity of cells was 17.78 and 14.33 respectively, i.e. an increase LDL-Dox accumulation was observed as the cells were treated with LPDS. After the cells were treated with LDL, the accumulation of LDL-Dox in the cells was less than that in cells treated with LPDS. The results suggest that most of the LDL-receptors might be bound by LDL or the expression level of LDL receptors was reduced in order to decrease the possibility of LDL-Dox bound to the receptor, thus there was a decreased of LDL-Dox accumulated in the cells treated with LDL when compared with those in the cells treated with LPDS. Moreover, the accumulation of LDL-Dox was increased in FC-treated cells when compared with that in cells treated with LPDS. The mean of the fluorescent intensity of cells under FC-treatment was 55.23 in which there was an increase of the LDL-Dox accumulation was observed when compared with that in cells treated with LPDS, i.e. the FC might up-regulate LDL receptors on the cells in order to increase the LDL-Dox accumulation. Moreover, the increase of fluorescent intensity in FC-treated cells did not due to the FC itself, mostly due to the fluorescent of Dox. As the cells were co-treated with FC and LDL, the accumulation of Dox in LDL-Dox treated cells was lower than that of the cells under FC-treatment only, but still higher than that when the cells were treated with LDL only. That means a part of LDL receptors up-regulated by FC might be bound by LDL, so the probability of

LDL-Dox bound to LDL receptors would be reduced. This phenomenon was comparable to the LDL receptor expression level under different kinds of the treatments.



Sample	Mean Fluorescence Value / units
Control	9.73
Control + LDL-Dox	14.33
LPDS + LDL-Dox	17.78
LDL + LDL-Dox	15.54
FC + LDL-Dox	55.23
FC and LDL + LDL-Dox	26.66

Fig. 3.16. The quantitative analysis of LDL-Dox accumulated in HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6-well plate overnight. Cells were treated with complete medium (control), lipoprotein deficient serum (LPDS), low density lipoprotein (LDL) in lipoprotein deficient serum, *Fructus Crataegus* (FC) in lipoprotein deficient serum and co-treatment of *Fructus Crataegus* and low density lipoprotein (FC and LDL) in lipoprotein deficient serum for 48 hours. After the pre-treatment, 1μM LDL-Dox mixing in lipoprotein deficient serum was added for 1 hour incubation. The accumulation of LDL-Dox was measured by flow cytometric analysis. The black line represented the control cells, i.e. cells without incubating with LDL-Dox. The red line represented the cells incubated with complete medium and 1μM LDL-Dox. The green line represented the cells incubated with lipoprotein deficient serum (LPDS) and 1μM LDL-Dox. The blue line represented the cells incubated with low density lipoprotein (LDL) and 1μM LDL-Dox. The purple line represented the cells incubated with *Fructus Crataegus* (FC) and 1μM LDL-Dox. The brown line represented the cells incubated with of *Fructus Crataegus* and low density lipoprotein (FC and LDL) and 1μM LDL-Dox.

3.1.2.7.3.Confocal laser scanning microscopic (CLSM) studies on the accumulation of LDL-Dox in HepG2 cells after *Fructus Crataegus* (FC) pre-treatment

As observed in Fig. 3.17 & 3.18, in HepG2 cells, the confocal laser scanning microscopic images of LPDS-treated cells and complete medium-treated cells showed that the LPDS-treated cells showed a higher fluorescent intensity than those obtained in the complete medium-treated cells. This result suggested that the intracellular level of LDL-Dox under LPDS treatment was higher than that under complete medium treatment. After the cells were treated with LDL, the fluorescent intensity of cells was lower than that treated with LPDS. It means the LDL could suppress LDL receptors expression or most of the LDL receptors was bound by LDL in order to decrease the intracellular level of LDL-Dox. When the cells were treated with FC, the fluorescent intensity of cells was higher than that of the cells treated with LPDS, i.e. the intracellular of LDL-Dox increased was due to the up-regulation of LDL receptors expression by FC and most of the fluorescent intensity of cells was due to the Dox, not due to the FC itself. But while the cells were co-treated with FC and LDL, the fluorescent intensity of cells was lower than that of the cells treated with FC-treated only, but still higher than that of the cells treated with LDL only. That means the power of up-regulation of LDL receptors by FC was higher than that of the level suppessed by LDL on the cells. Thus, the intracellular concentration of LDL-Dox under the co-treatment was higher than that of the cells treated with LDL. This phenomenon was comparable to the LDL-Dox accumulation in the cells under different kinds of treatments.

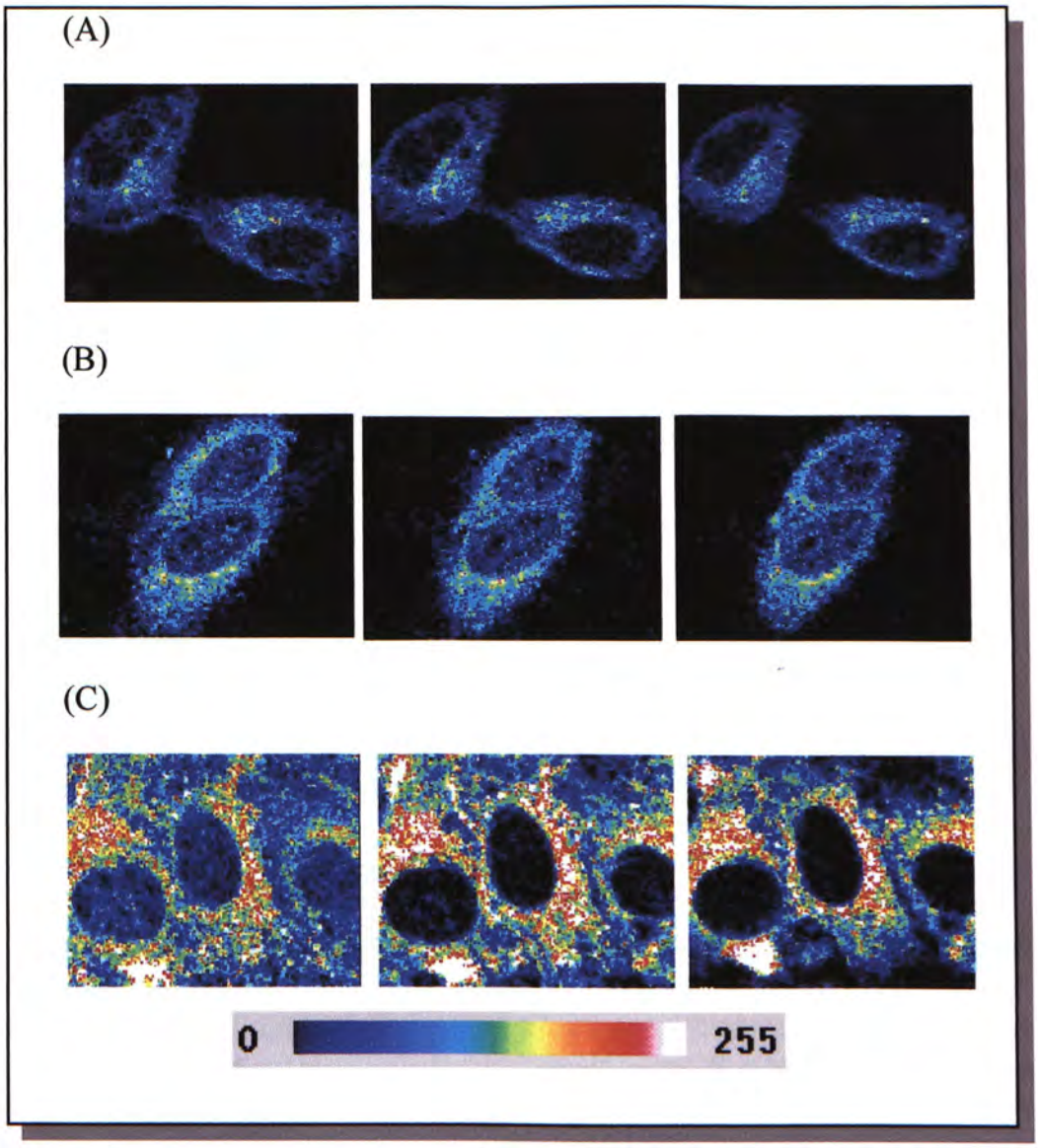


Fig. 3.17. The confocal laser scanning microscopic analysis on the accumulated level of LDL-Dox in HepG2 cells. Cells at 5 x 10^4 cells were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM LDL-Dox for 1 hour at 37°C. (A), complete medium (control)-treated HepG2 cells were scanned along the Z-axis for 1μm. (B), lipoprotein deficient serum (LPDS)-treated HepG2 cells were scanned along Z-axis for 1μm. (C), *Fructus Crataegus* (FC) in LPDS-treated HepG2 cells were scanned along Z-axis for 1μm.

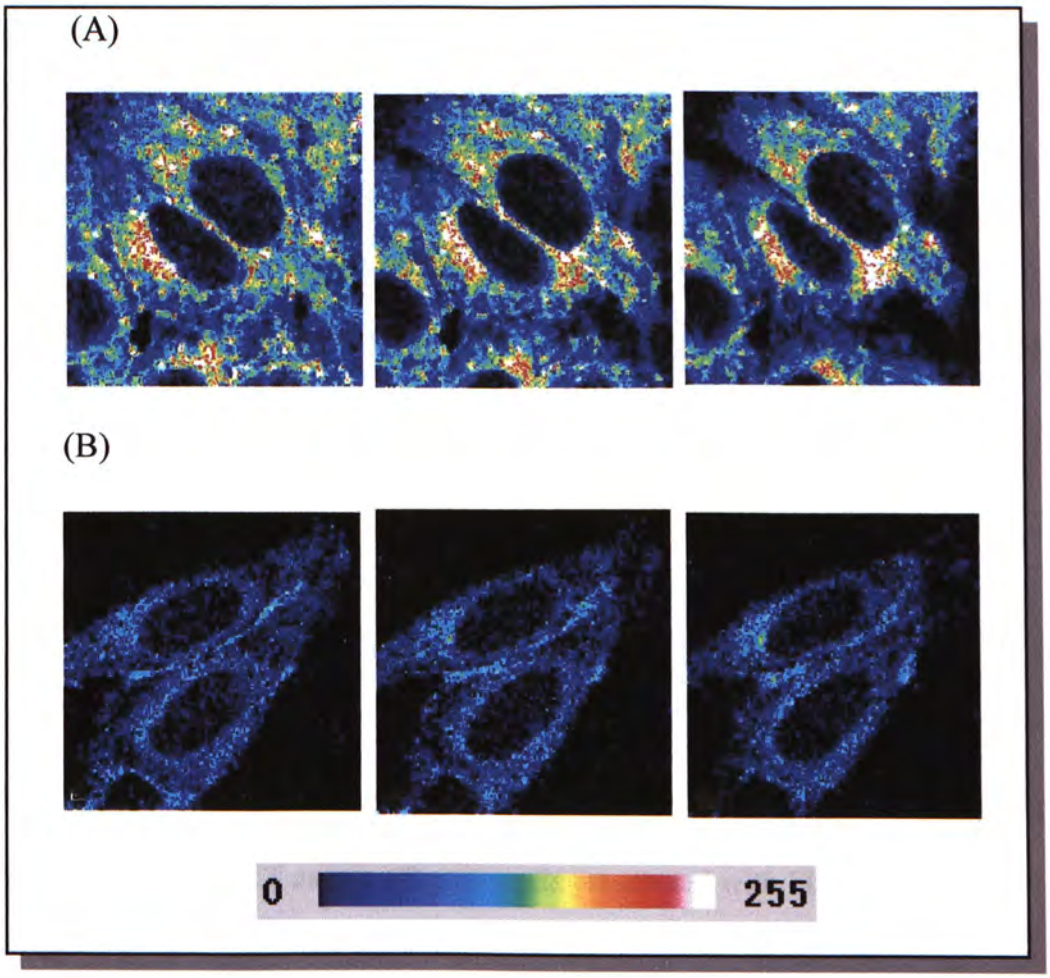


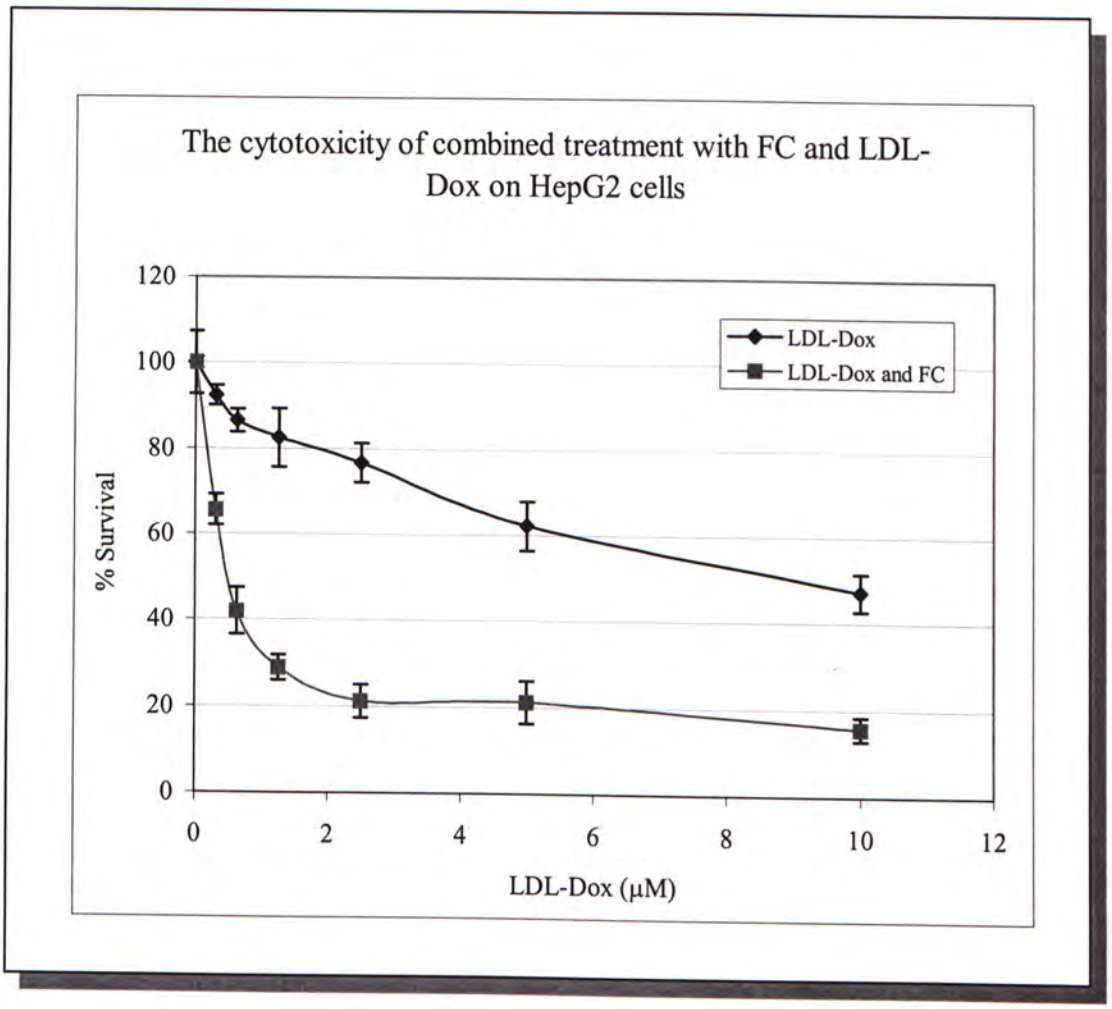
Fig. 3.18. The confocal laser scanning microscopic analysis on the accumulated level of LDL-Dox in HepG2 cells. Cells at 5 x 10^4 cells were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM LDL-Dox in LPDS for 1 hour at 37°C. (A), co-treaed with *Fructus Crataegus* and low density liporotein (FC and LDL) in LPDS on HepG2 cells were scanned along the Z-axis for 1μm. (B), low density lipoprotein in LPDS-treated HepG2 cells were scanned along Z-axis for 1μm.

3.1.2.7.4.Cytotoxicity of combined treatment with LDL-Dox and *Fructus Crataegus* (FC)

Since the results of the protein expression level of LDL receptors and the accumulation of LDL-Dox in the HepG2 cells was increased under FC-treatment, the percentage of cell survival under the combined treatment of FC and LDL-Dox might be different from the cells treated with LDL-Dox only.

The HepG2 cells were pre-treated with FC in LPDS medium up to 48 hours, then the cells were incubated at different concentrations of LDL-Dox ranging from 10μM to 0.3125μM under 37°C, 5% CO_2 for 24 hours and the cells viability was determined by MTT assay. After the cells pre-treated with FC, the IC_{50} of LDL-Dox was 0.6μM which was about 15-fold lower than that under LDL-Dox treated only, the IC_{50} of LDL-Dox after 24 hours treatment without FC-treated was 8.9μM (Fig. 3.19). Moreover, there was not any cytotoxic effect of 1mg/ml FC on HepG2 cells observed in the previous studies, thus the cytotoxicity of HepG2 cells determined in this assay was due to the cytotoxic effect of LDL-Dox.

The results suggested that after FC pre-treatment, the LDL receptor expression level was increased, which could enhance the LDL-Dox accumulated in the cells in order to increase the cytotoxic effect of LDL-Dox.



Sample	The value of IC_{50}
LDL-Dox only	8.9
LDL-Dox and FC-treatment	0.6

Fig. 3.19. Effect of combined treatment with low density lipoprotein-doxorubicin and the pre-treatment of *Fructus Crataegus* (FC) on the survival of HepG2 cells. Cells at 5 x 10^3 cells/well were seeded in a 96 wells plate overnight. Cells were treated with 1mg/ml of FC in lipoprotein deficient serum for 48 hours. After 48 hours, the cells were washed with 1X PBS twice, then the cells were incubated at different concentrations of LDL-Dox for further 24 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.

3.1.3. Studies on multidrug human resistant hepatoma cell line (R-HepG2 cells)

3.1.3.1. The overexpression level of P-glycoprotein in a resistant cell line R-HepG2

One phenomenon of multidrug resistant cell lines is the over-expression of P-glycoprotein. In R-HepG2 cells, they also had a higher expression level of P-glycoprotein when compared with HepG2 cells (Fig. 3.20). Therefore the IC_{50} of Dox treated on HepG2 cells were usually lower than that treated on R-HepG2 cells.

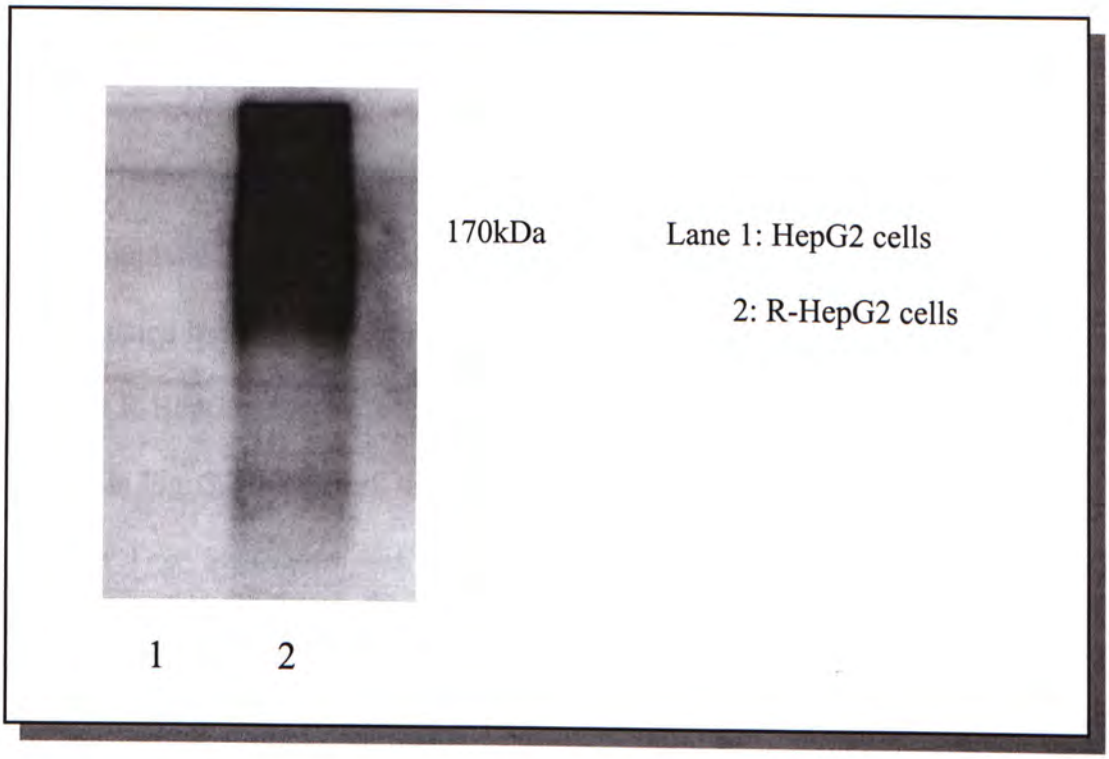
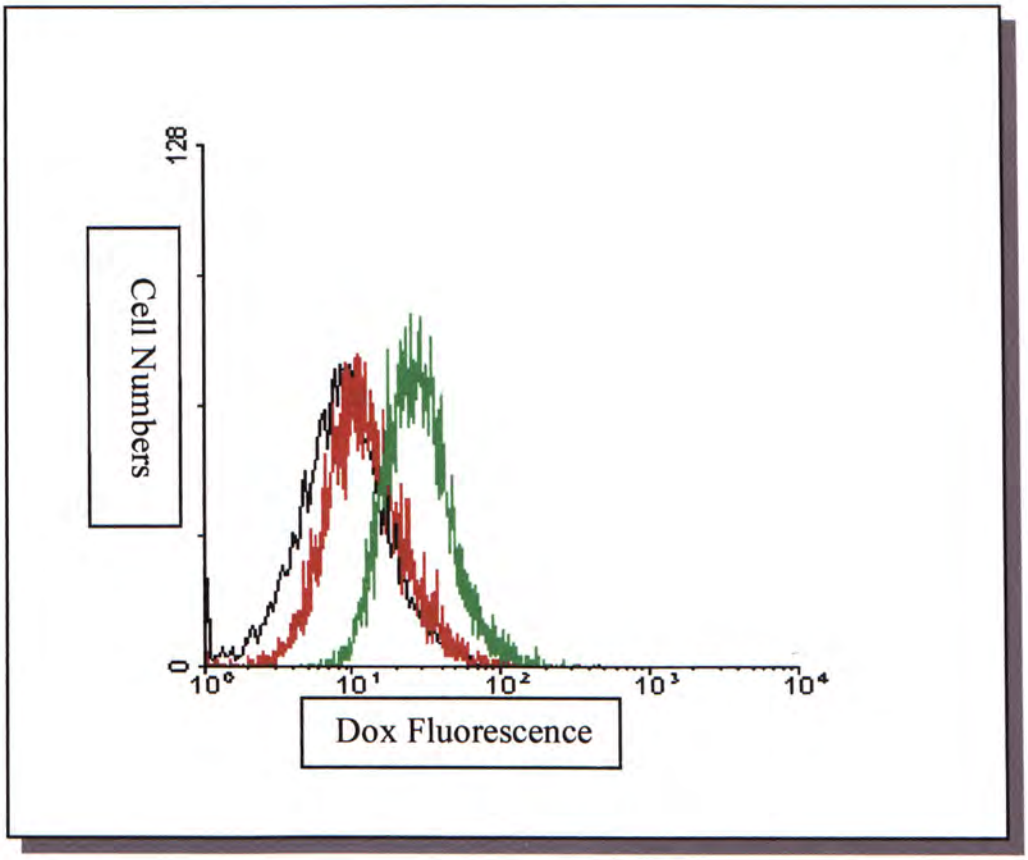


Fig. 3.20. The expression level of P-glycoprotein in HepG2 cells and R-HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6 well-plate for 48 hours. The level of P-glycoprotein was analyzed by Western blot analysis. Aliquot of 25μg of protein was loaded in each lane.

3.1.3.2.The comparison of Dox and LDL-Dox accumulated in R-HepG2 cells

Since the higher expression level of P-glycoprotein in R-HepG2 cells, Dox might be easily pumped out from the cells, i.e. the concentration of Dox accumulated in the cells might be decreased in order to reduce the cytotxic effect of Dox on R-HepG2 cells. This hypothesis was examined in the present experiment. As shown in Fig. 3.21, the mean of the fluorescent intensity of R-HepG2 cells with and without Dox incubation was 10.46 and 8.98 respectively. There was no clear difference between the cells incubated with Dox or not. Moreover, the expression level of low density lipoprotein receptor on cancer cells was higher than that of normal cells, thus the accumulation of LDL-Dox was higher than that of Dox in R-HepG2 cells. The mean of the fluorescent intensity of cells on LDL-Dox treatment was 25.71. There was an increase of fluorescent intensity of cells treated with LDL-Dox when compared with the cells treated with Dox. The comparison of Dox and LDL-Dox accumulated in R-HepG2 cells was also performed (Fig. 3.21).



Sample	Mean Fluorescence Value / units
Control	8.98
Dox	10.46
LDL-Dox	25.71

Fig. 3.21. The quantitative analysis of Dox and LDL-Dox accumulated in R-HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6-well plate for 48 hours. Cells were treated with 1μM Dox in complete medium or LDL-Dox in lipoprotein deficient serum for 1 hour. The accumulation of Dox and LDL-Dox was measured by flow cytometric analysis. The black line represented the control cells, i.e. cells incubated without Dox or LDL-Dox. The red line represented the cells incubated with 1μM Dox. The green line represented the cells incubated with 1μM LDL-Dox.

3.1.3.3.Confocal laser scanning microscopic (CLSM) studies on the accumulation of Dox and LDL-Dox in R-HepG2 cells

Since the size of Dox was smaller than the pore size of P-glycoprotein, most of the Dox could be pumped out by the P-glycoprotein in R-HepG2 cells. From the results of confocal laser scanning microscopic images of Dox-treated cells and LDL-Dox-treated cells, it showed that the cells treated with LDL-Dox exhibited a higher degree of fluorescent intensity than that with Dox. Moreover, the Dox accumulated in the cells was located near the cell membrane (Fig. 3.22).

This result suggested that the intracellular level of LDL-Dox was higher than that of Dox which was comparable to the result of the accumulation of LDL-Dox and Dox in the cells.

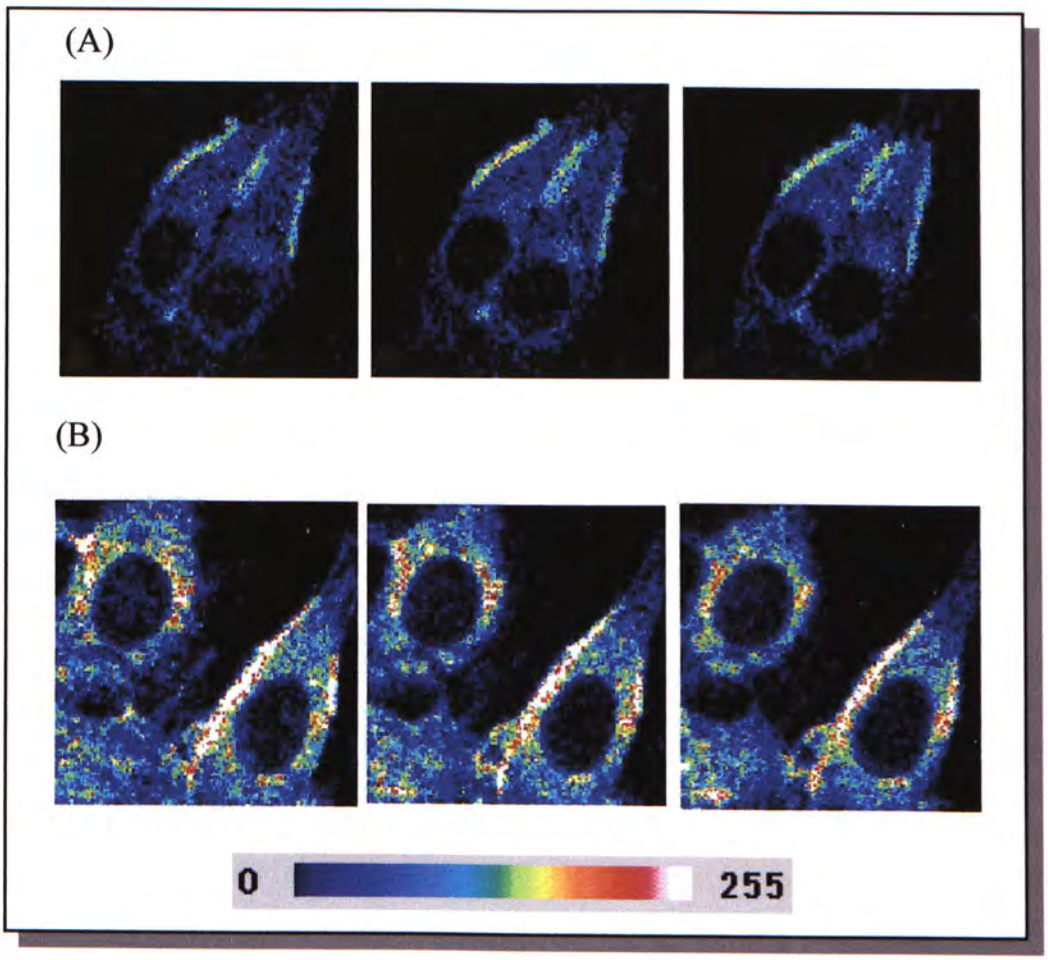


Fig. 3.22. The confocal laser scanning microscopic analysis on the accumulated level of Dox and LDL-Dox in R-HepG2 cells. Cells at 5 x 10^4 cells were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM LDL-Dox for 1 hour at 37°C, 5% CO_2 for 1 hour. (A), Dox-treated R-HepG2 cells were scanned along the Z-axis for 1μm. (B), LDL-Dox-treated R-HepG2 cells were scanned along Z-axis for 1μm.

3.1.3.4.The comparsion of the cytotoxicity of Dox and LDL-Dox on R-HepG2 cells

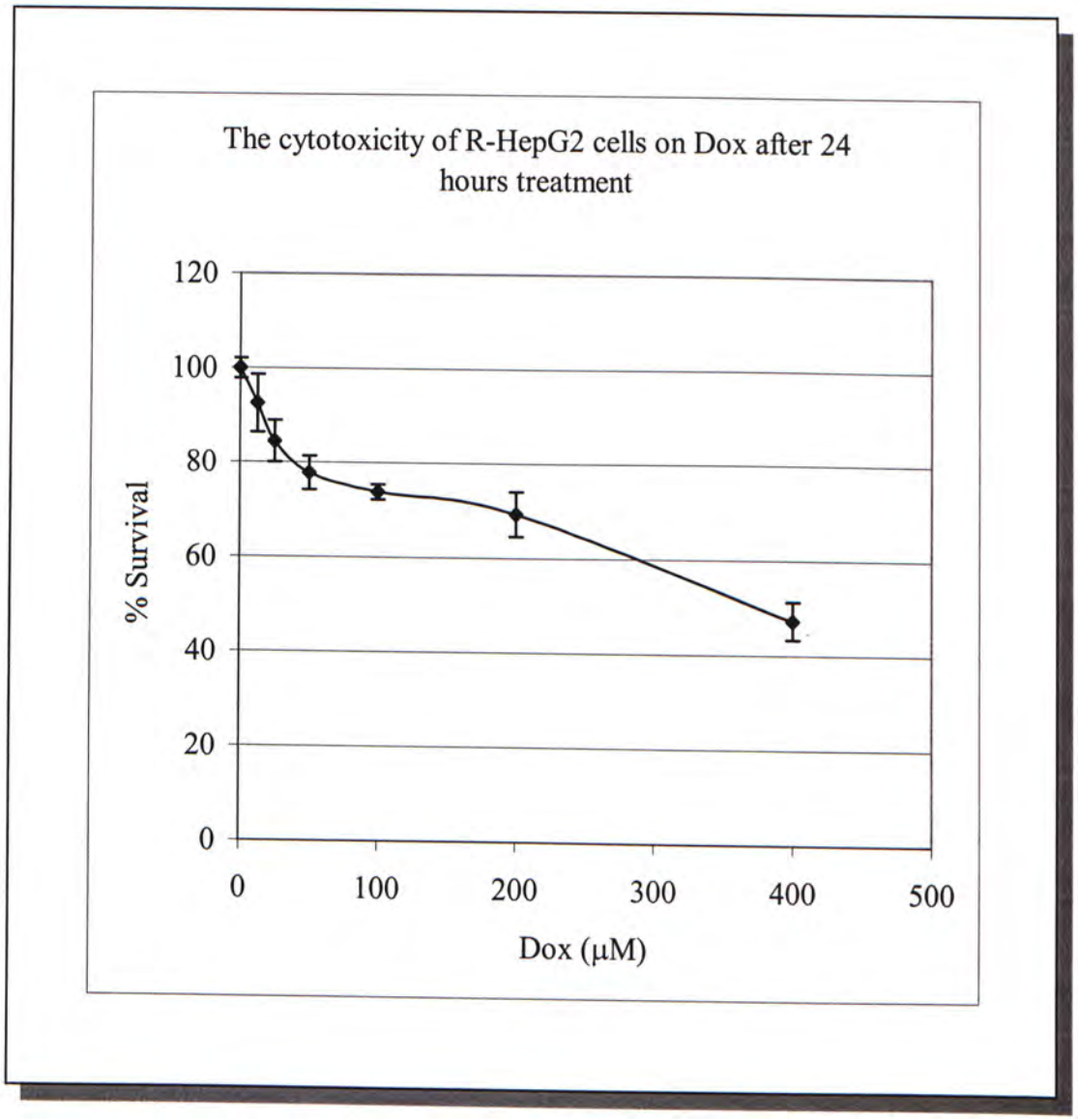
According to the measurement of Dox accumulated in R-HepG2 cells, the P-glycoprotein existing might reduce the concentration of Dox accumulated in the cells resulting in a decrease in the cytotoxic effect of Dox. This possibility was raised due to the ability to incubate the cells at a higher concentrations of Dox than that on HepG2 cells. The concentrations of Dox and LDL-Dox ranging from 400μM to 12.5μM and 50μM to 1.5626μM respectively were needed to incubate R-HepG2 cells under 37°C, 5% CO_2 for 24 hours, 48 hours and 72 hours. The cells survival was determined by MTT assay.

When the cells were incubated with drugs for 24 hours, the IC_{50} of Dox and LDL-Dox were found to be 368μM (Fig. 3.23) and 48μM (Fig. 3.24) respectively. In addition, when the incubation time prolonged from 24 hours to 48 hours, the IC_{50} of Dox and LDL-Dox were found to be 300μM (Fig. 3.25) and 15μM (Fig. 3.26) respectively. After 72 hours incubation, IC_{50} of Dox and LDL-Dox were found to be 240μM (Fig. 3.27) and 7μM (Fig. 3.28) respectively.

These results showed that as the concentration of Dox and LDL-Dox increased, the numbers of cell viability were decreased, i.e. the percentage of cell survival was dose-dependent (Fig. 3.29). Also, when the incubation time was prolonged, cell viability was decreased, i.e. the percentage of cells survival was time-

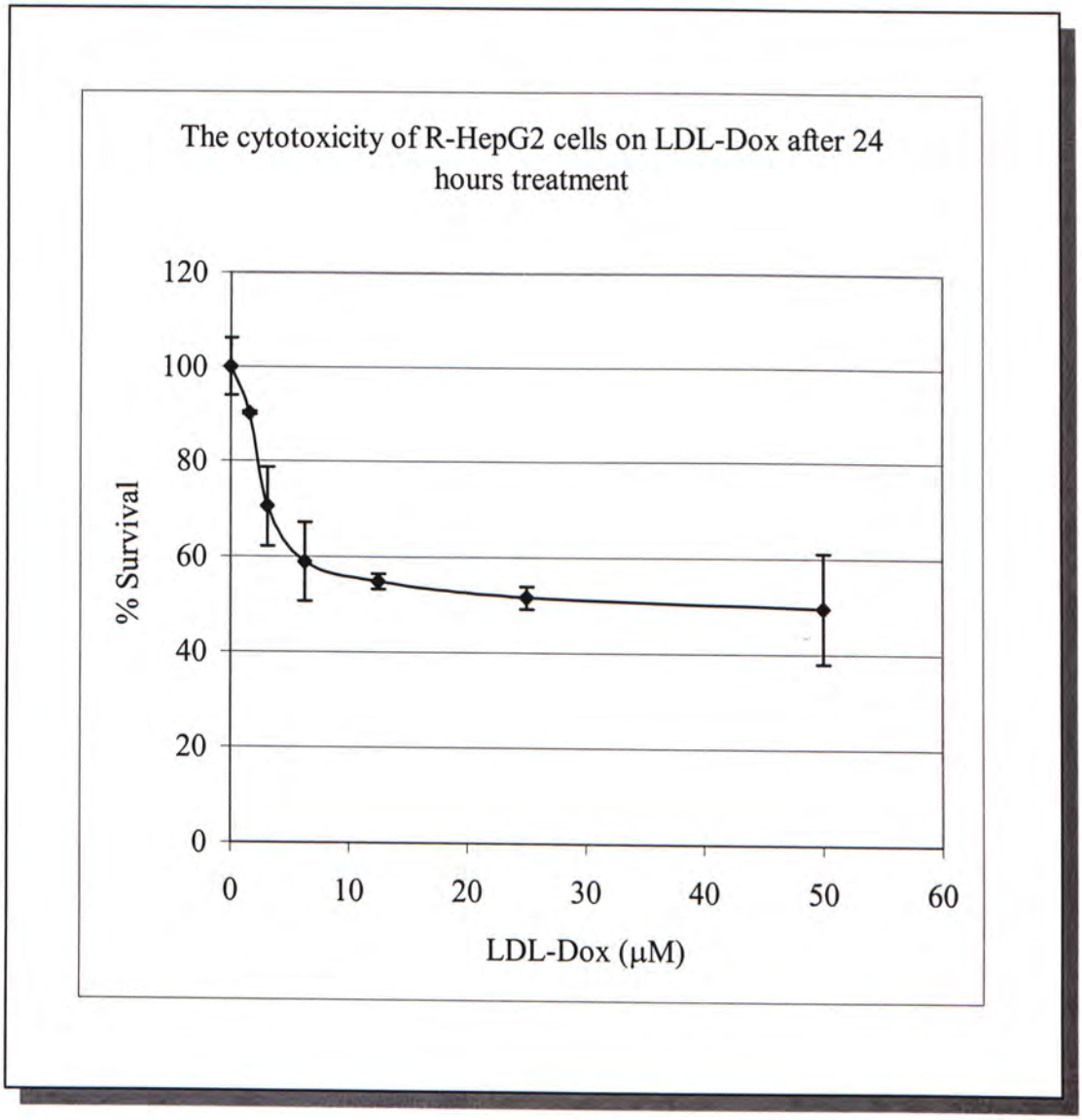
dependent (Fig. 3.30). Hence, the percentage survival of R-HepG2 cells was time- and dose- dependent on Dox and LDL-Dox.

In addition, the IC_{50} of LDL-Dox was usually lower than that of Dox at different time courses. This might indicate that after Dox coupled into LDL, the complex was not easily pumped out by the P-glycoprotein in R-HepG2 cells so that the cytotoxic effect of LDL-Dox in R-HepG2 cells could be enhanced.



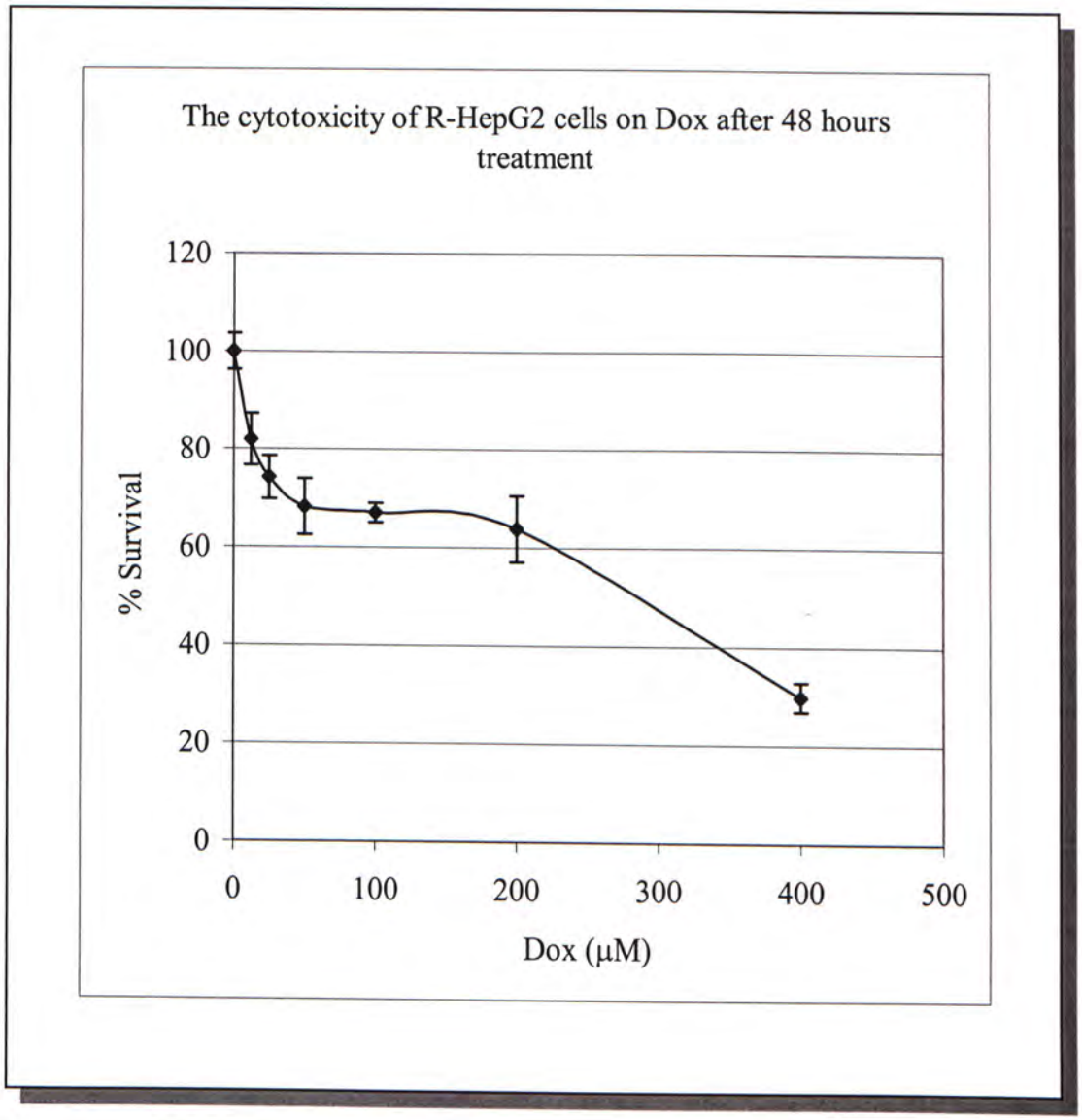
Sample	The value of IC_{50}
Dox	368

Fig. 3.23. Effect of doxorubicin on the survival of R-HepG2 cells after 24 hours incubation. Cells at 2 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of Dox for 24 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



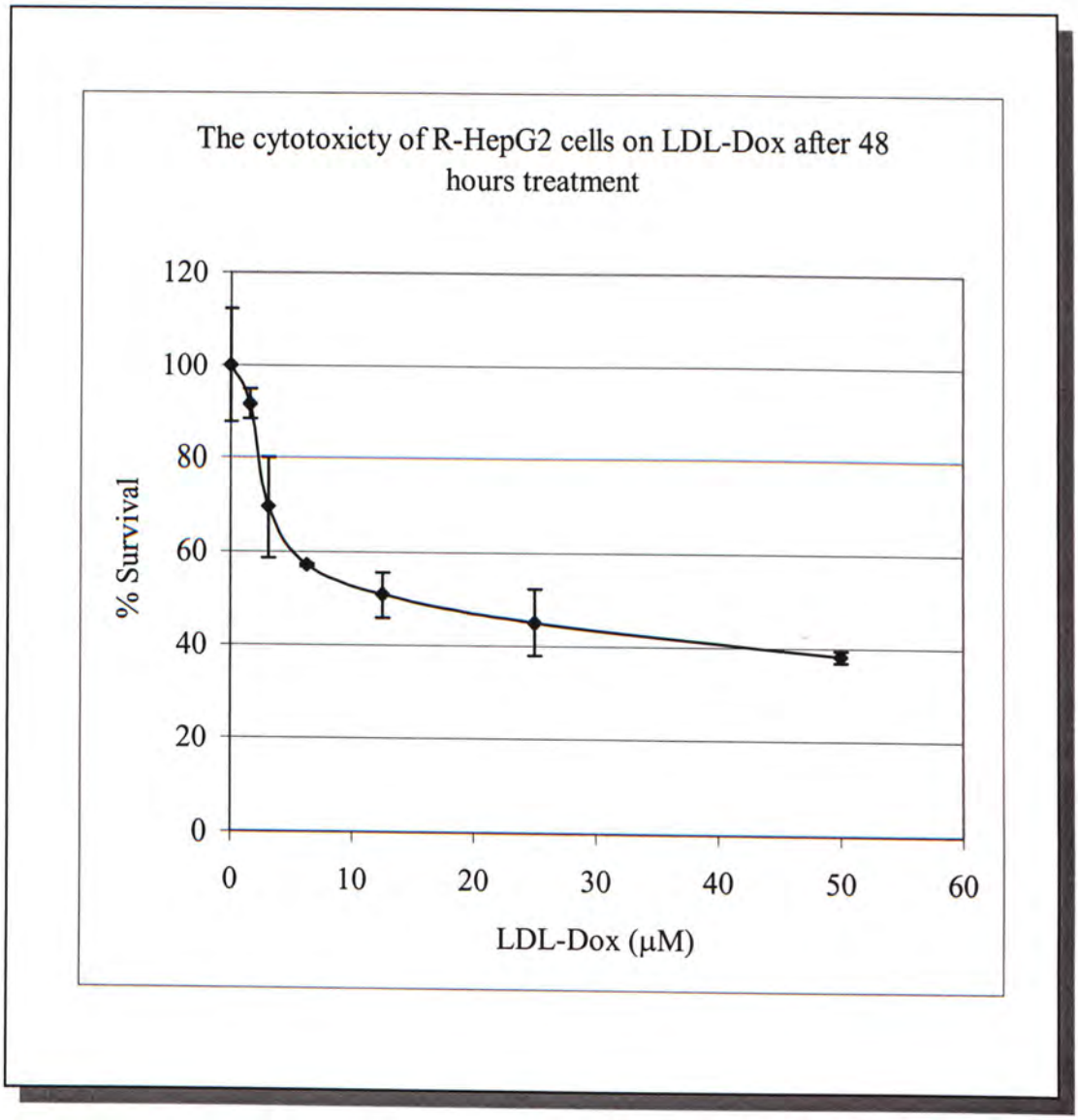
Sample	The value of IC_{50}
LDL-Dox	48

Fig. 3.24. Effect of low density lipoprotein-doxorubicin on the survival of R-HepG2 cells after 24 hours incubation. Cells at 2 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of LDL-Dox for 24 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



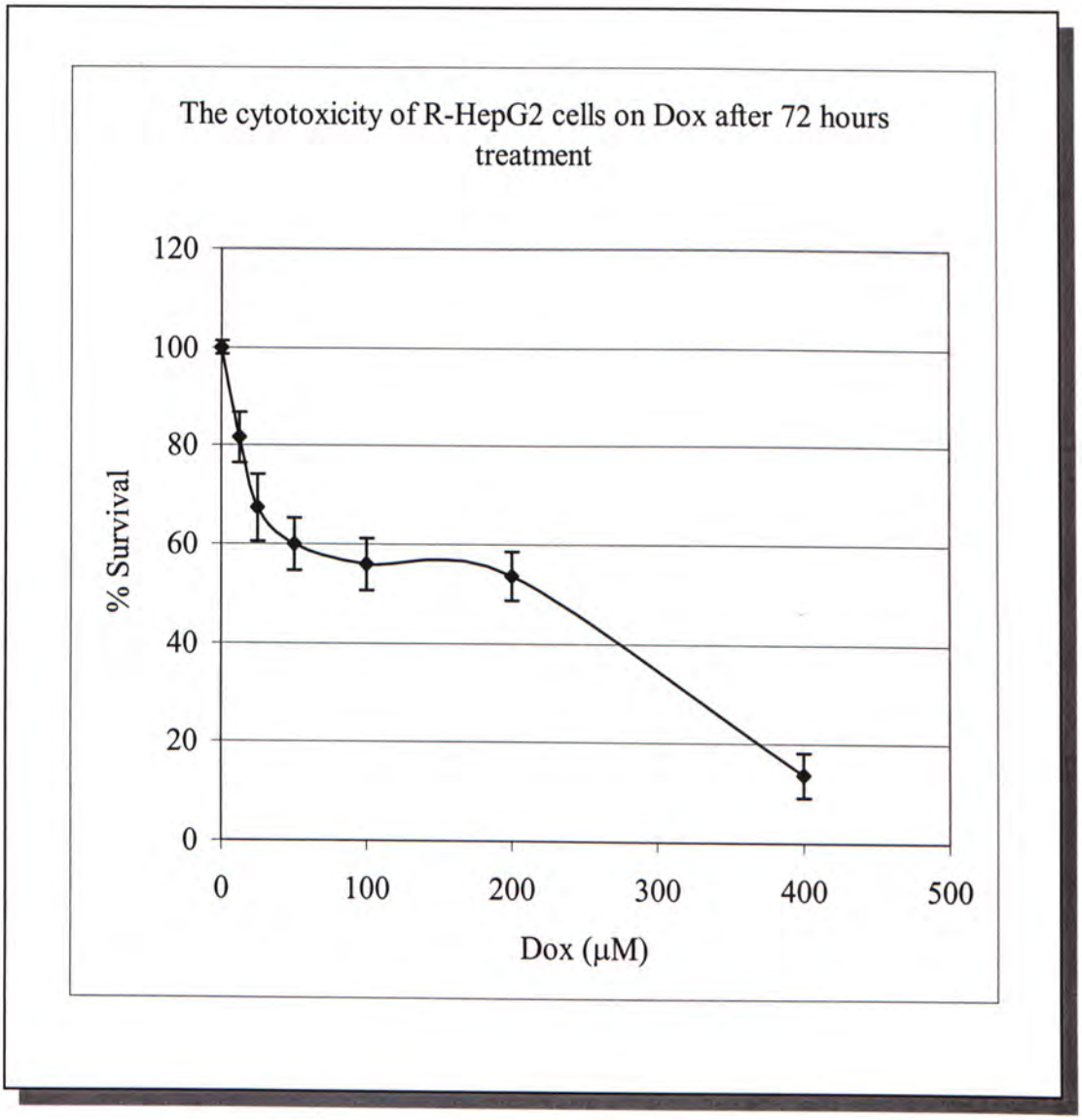
Sample	The value of IC_{50}
Dox	300

Fig. 3.25. Effect of doxorubicin on the survival of R-HepG2 cells after 48 hours incubation. Cells at 1 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of Dox for 48 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



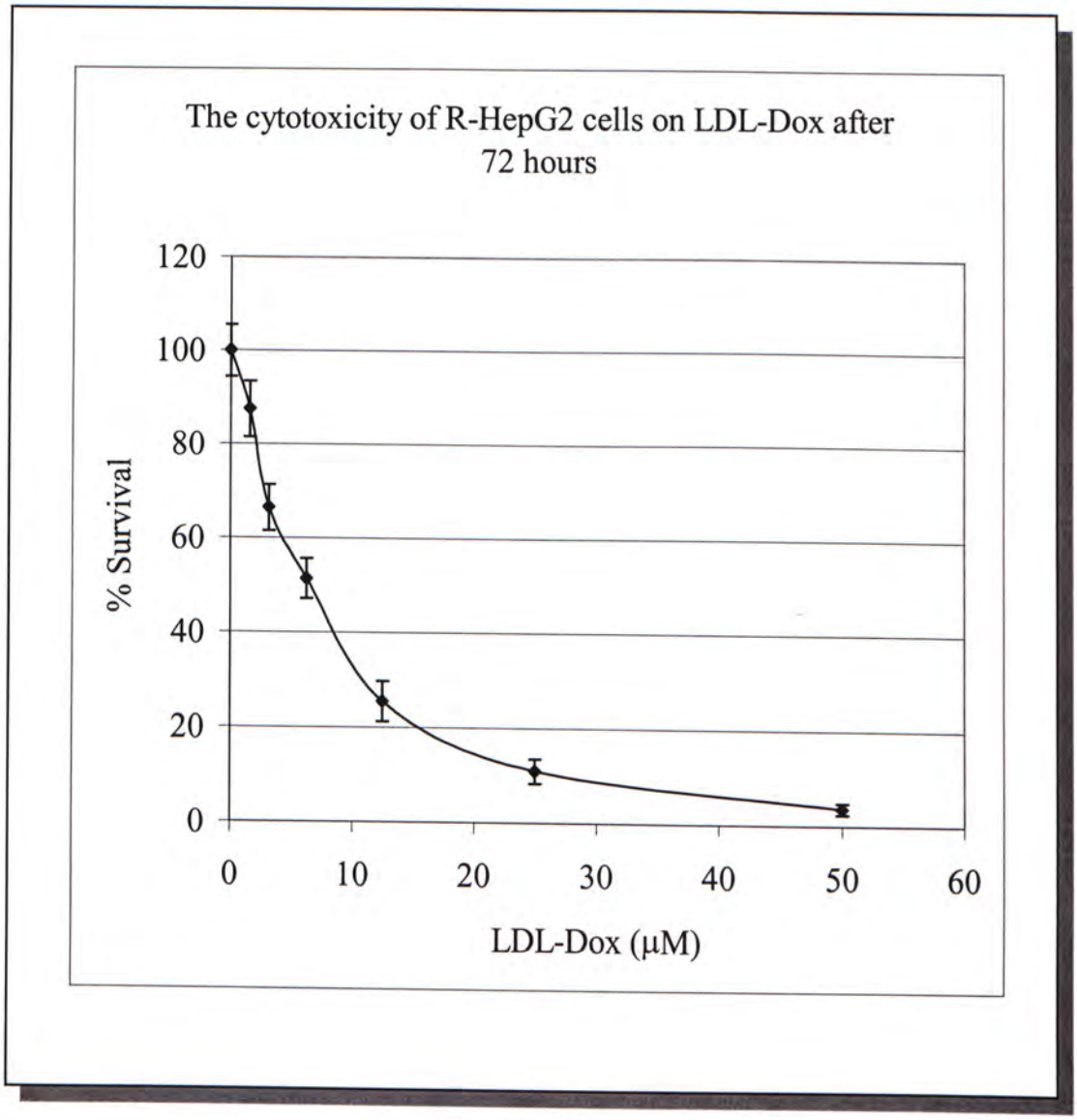
Sample	The value of IC_{50}
LDL-Dox	15

Fig. 3.26. Effect of low density lipoprotein-doxorubicin on the survival of R-HepG2 cells after 48 hours incubation. Cells at 1 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of LDL-Dox for 48 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



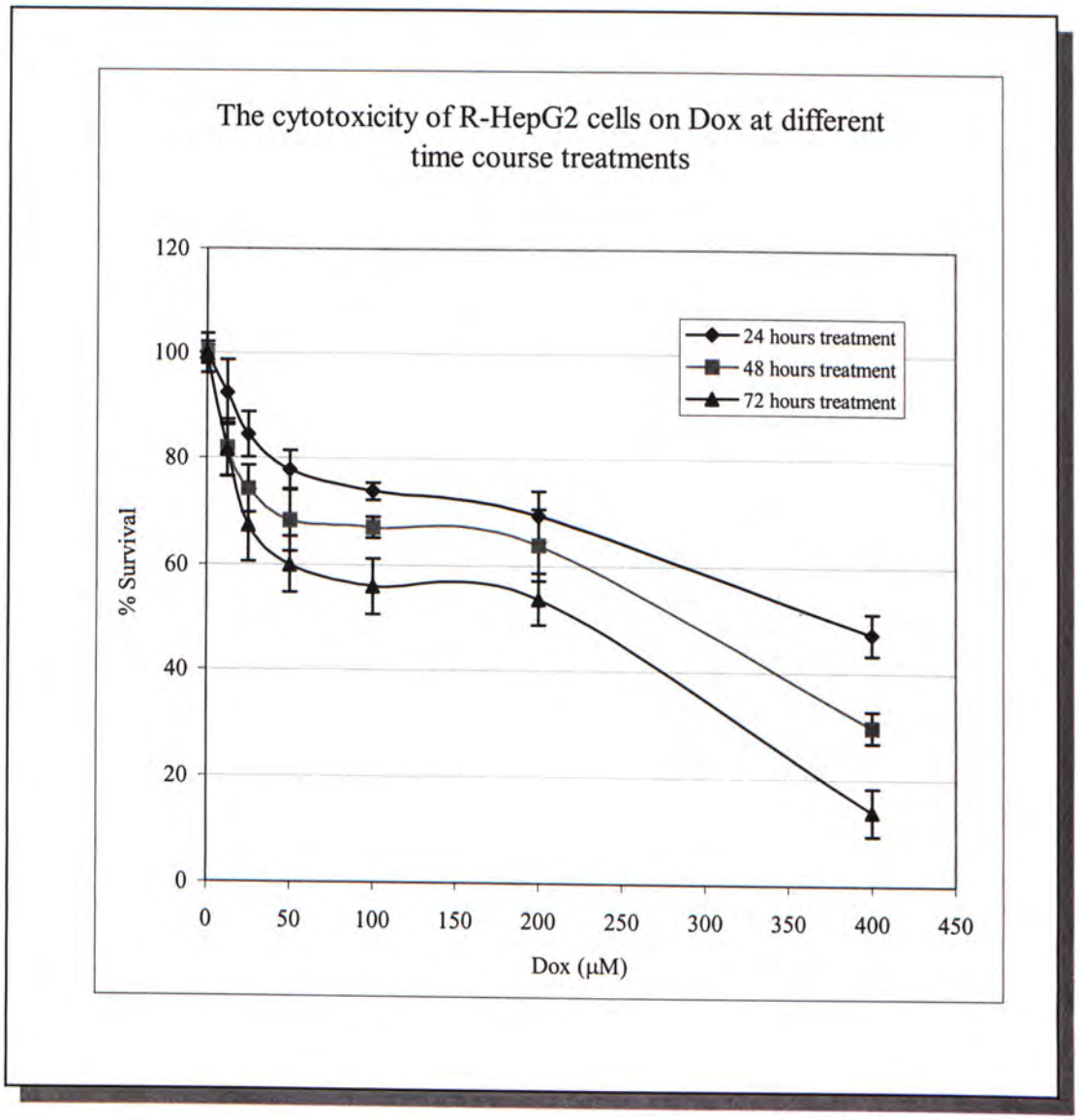
Sample	The value of IC_{50}
Dox	240

Fig. 3.27. Effect of doxorubicin on the survival of R-HepG2 cells after 72 hours incubation. Cells at 3 x 10^5 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of Dox for 72 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



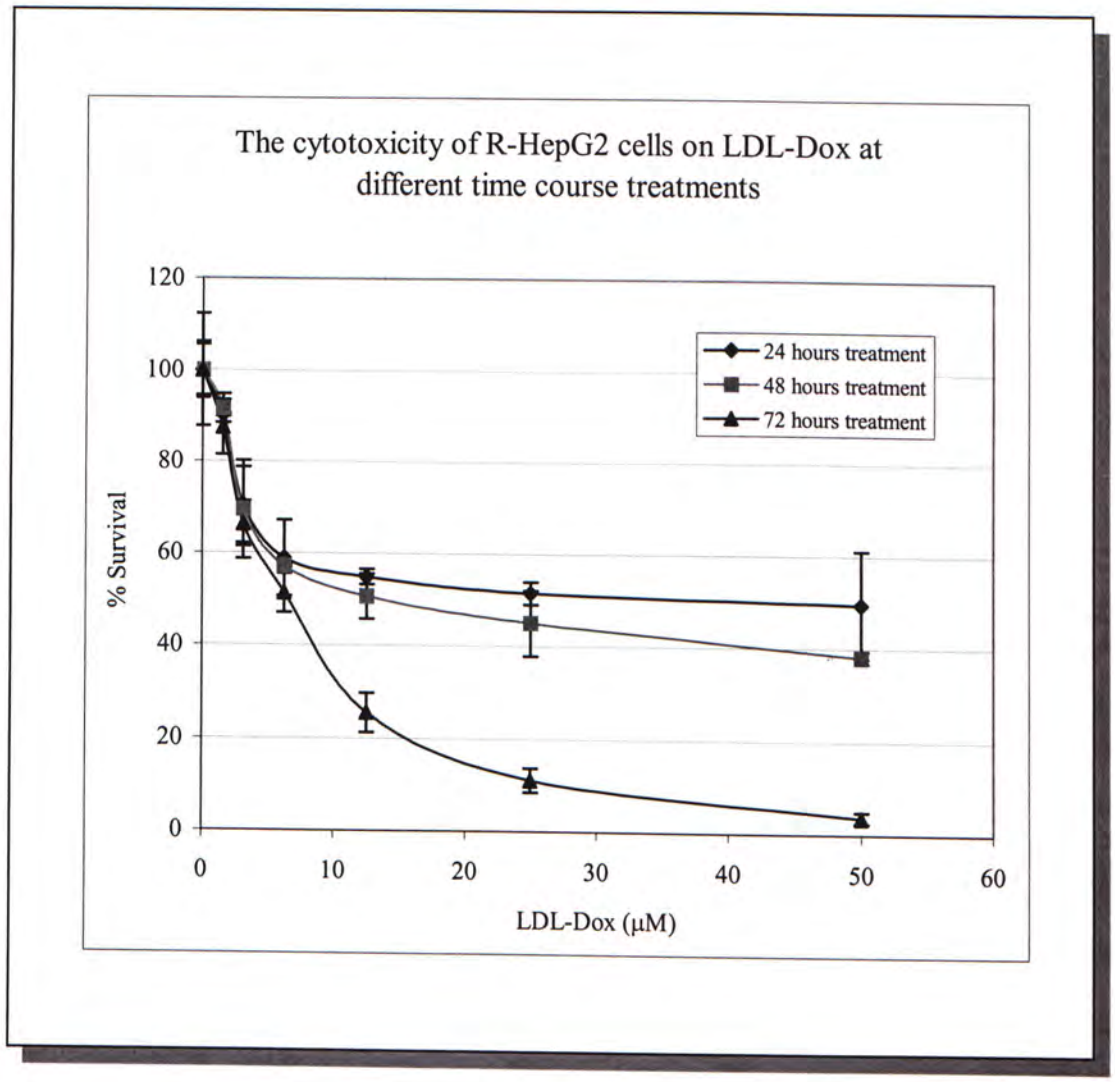
Sample	The value of IC_{50}
LDL-Dox	7

Fig. 3.28. Effect of low density lipoprotein-doxorubicin on the survival of R-HepG2 cells after 72 hours incubation. Cells at 3 x 10^5 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of LDL-Dox for 72 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



Time course	The value of IC_{50} on Dox
24 hours	368
48 hours	300
72 hours	240

Fig. 3.29. Effect of doxorubicin on the survival of R-HepG2 cells at different time course treatments. Cells at 2 x 10^4, 1 x 10^4 and 3 x 10^5 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of doxorubicin for 24, 48 and 72 hours respectively. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



Time course	The value of IC_{50} on LDL-Dox
24 hours	48
48 hours	15
72 hours	7

Fig. 3.30. Effect of low density lipoprotein-doxorubicin on the survival of R-HepG2 cells at different time course treatments. Cells at 2 x 10^4, 1 x 10^4 and 3 x 10^5 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of LDL-Dox for 24, 48 and 72 hours respectively. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.

3.1.3.5.The comparison of the cytotoxicty of Dox and LDL-Dox with and without hyperthermia on R-HepG2 cells

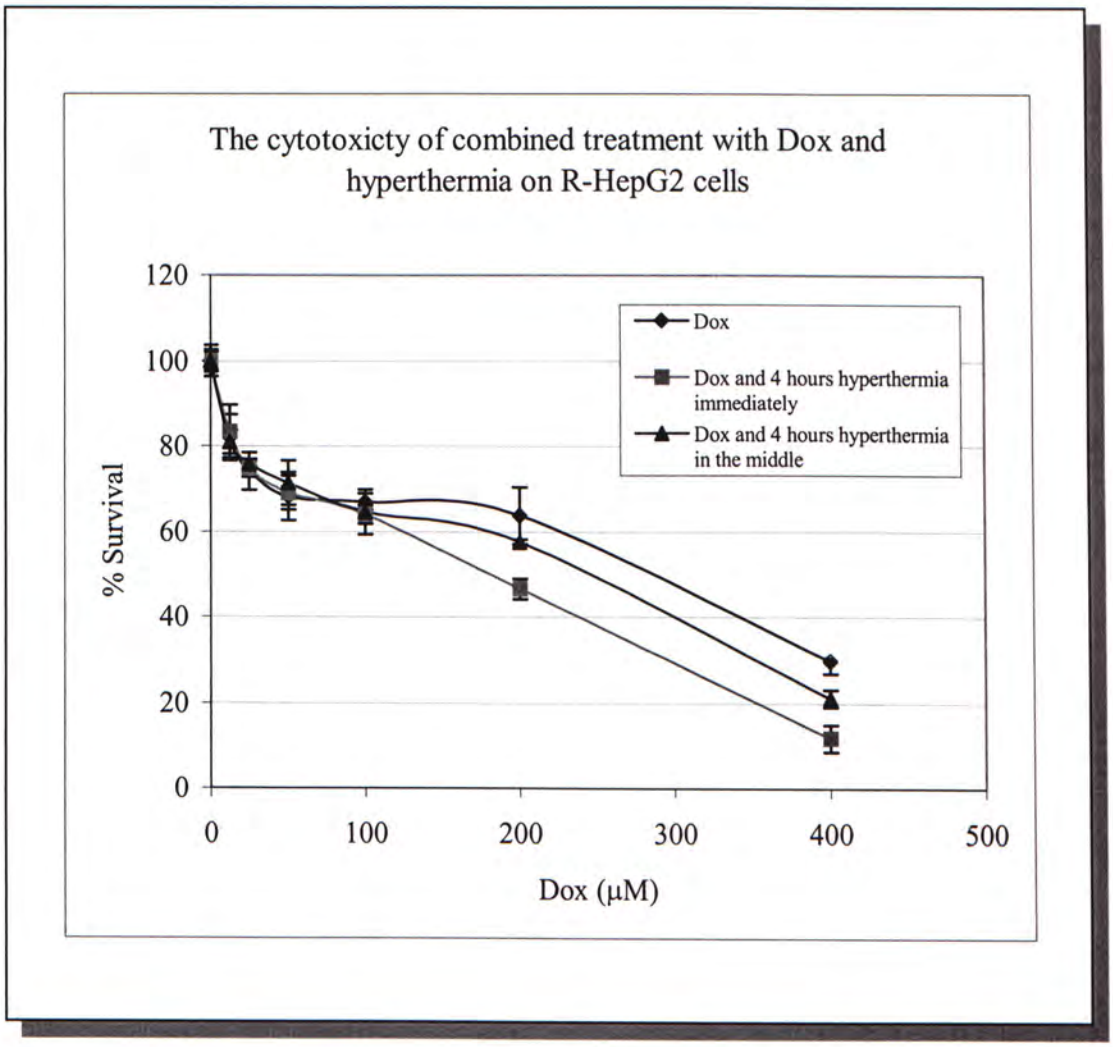
In R-HepG2 cells, after the cells were incubated at 43°C at different time periods for 4 hours, and the hyperthermia was then applied at the beginning of drug treatment, i.e. at 0 hour to 4 hours during the drug treatment, or in the middle of the drug treatment, i.e. at 22 hours to 26 hours drug the drug treatment. The cytotoxic effect of Dox and LDL-Dox was enhanced by hyperthermia.

Moreover for the Dox treatment, the IC_{50} of cells incubated with and without hyperthermia at 43°C were 300μM, 180μM and 254μM respectively (Fig. 3.31). The effect of hyperthermia at the beginning of Dox treatment was higher than that in the middle of the drug treatment and their IC_{50} were 180μM and 254μM respectively.

For LDL-Dox treatment, the IC_{50} of cells incubated with and without hyperthermia at 43°C were 15μM, 6.2μM and 8.9μM respectively (Fig. 3.32). The cytotoxic effect of LDL-Dox combined with hyperthermia at the beginning of LDL-Dox treatment was higher than that in the middle of the drug treatment and their IC_{50} were 6.2μM and 8.9μM respectively.

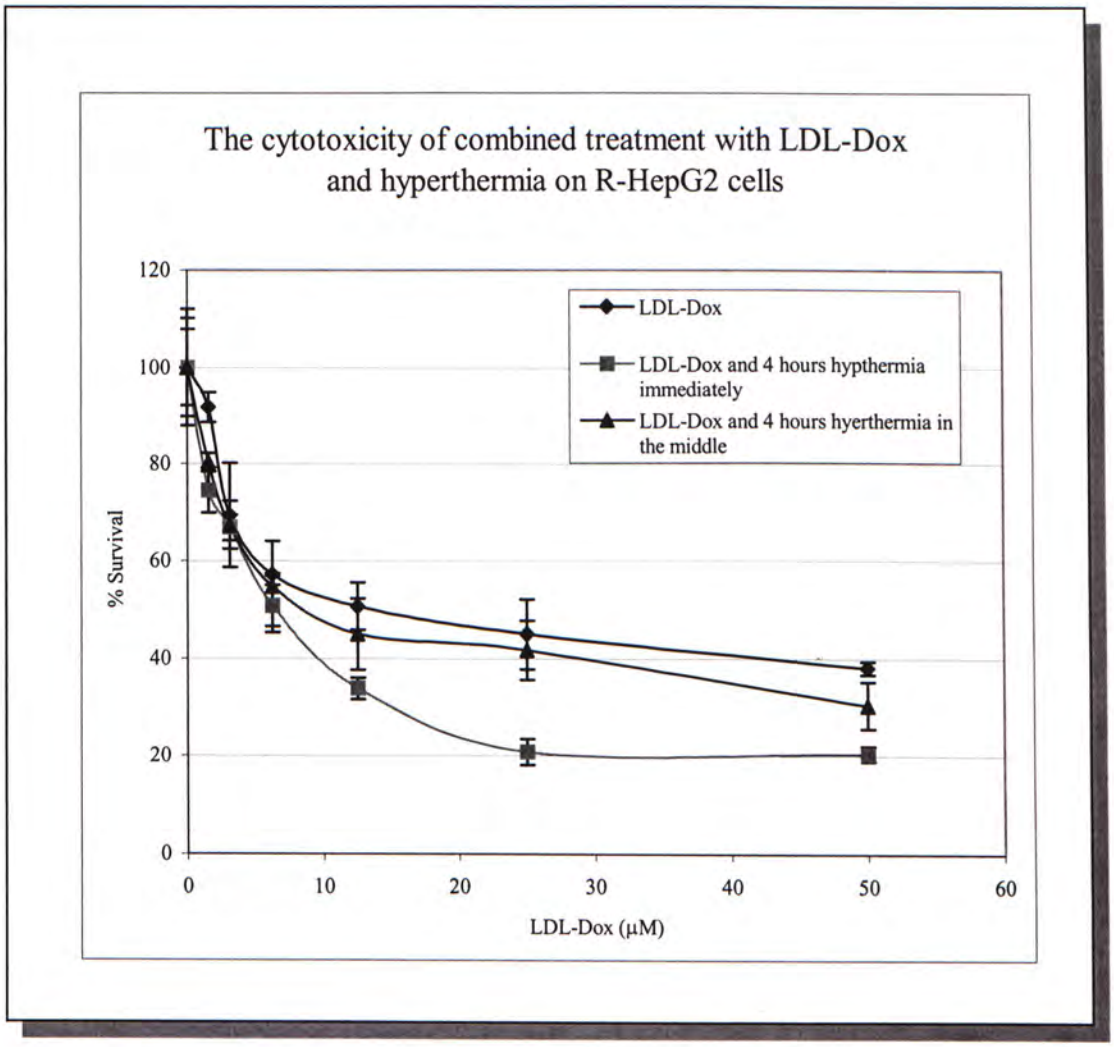
The result suggested that the hyperthermia effect could enhance the cytotoxic effect of Dox and LDL-Dox. Furthermore, the effect of hyperthermia applied at the beginning of the drug treatment was greater than that observed when

htperthermia was applied in the middle of the drug treatment, and this phenomenon could be observed at both Dox and LDL-Dox treatment.



Sample	The value of IC_{50}
Dox only	300
Dox and hyperthermia immediately	180
Dox and hyperthermia in the middle	245

Fig. 3.31. Effect of combined treatment with doxorubicin and hyperthermia on the survival of R-HepG2 cells. Cells at 1 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of Dox for 48 hours. During the incubation time, the hyperthermia was applied immediately after adding drugs, i.e. at 1 hour to 4 hours of the drug treatment, or in the middle of the drug treatment, i.e. at 22 hours to 26 hours of drug treatment. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.



Sample	The value of IC_{50}
LDL-Dox only	15
LDL-Dox and hyperthermia immediately	6.2
LDL-Dox and hyperthermia in the midd	8.9

Fig. 3.32. Effect of combined treatment with low density lipoprotein-doxorubicin and hyperthermia on the survival of R-HepG2 cells. Cells at 1 x 10^4 cells/well were seeded in a 96 wells plate overnight. Cells were treated with different concentrations of LDL-Dox for 48 hours. During the incubation time, the hyperthermia was applied immediately after adding drugs, i.e. at 1 hour to 4 hours of the drug treatment, or in the middle of the drug treatment, i.e. at 22 hours to 26 hours of the drug treament. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.

3.1.3.6.The comparison of the accumulation of Dox and LDL-Dox in R-HepG2 cells treated in combination with hyperthermia

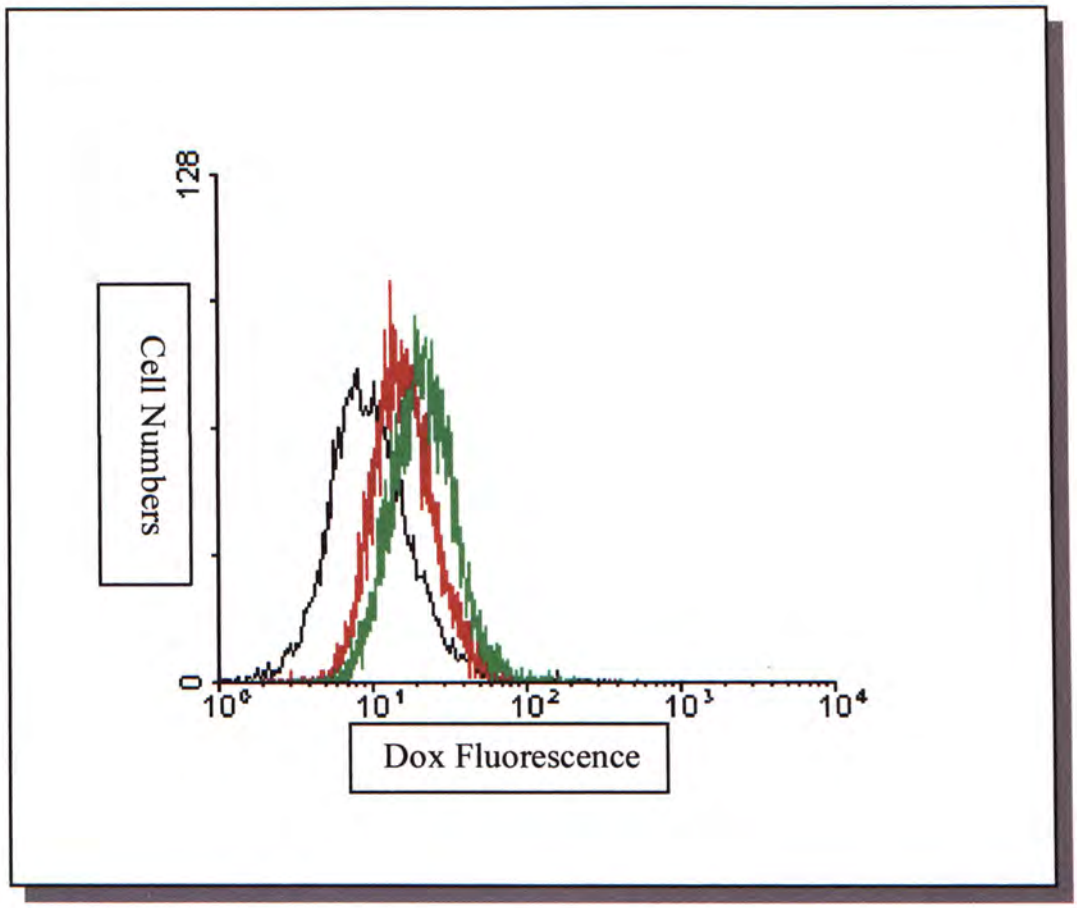
According to the results of Dox and LDL-Dox combined with hyperthermia, hyperthermia could enhance the cytotoxic effect of Dox and LDL-Dox. Whether this effect was enhanced by the accumulation of Dox and LDL-Dox in the cells would be examined by flow cytometric analysis.

When comparing between the accumulations of 1μM of Dox in the R-HepG2 cells incubated at 37°C and 43°C both at 5% CO_2 1 hour, the mean of the fluorescent intensity of cells incubated at 37°C was 12.52 and the mean of the cells incubated at 43°C was 21.88. There was an increase in the accumulation of Dox into the cells when comparing with the hyperthermia and control cells (Fig. 3.33).

In addition, when comparing between the accumulations of 1μM of LDL-Dox in the R-HepG2 cells incubated at 37°C and 43°C both at 5%CO_2 for 1 hour, the mean of the fluorescent intensity of cells incubated at 37°C was 27.13 and the cells incubated at 43°C was 27.59. There was not a clear difference in the accumulation of LDL-Dox between the cells under 37°C and 43°C (Fig. 3.34).

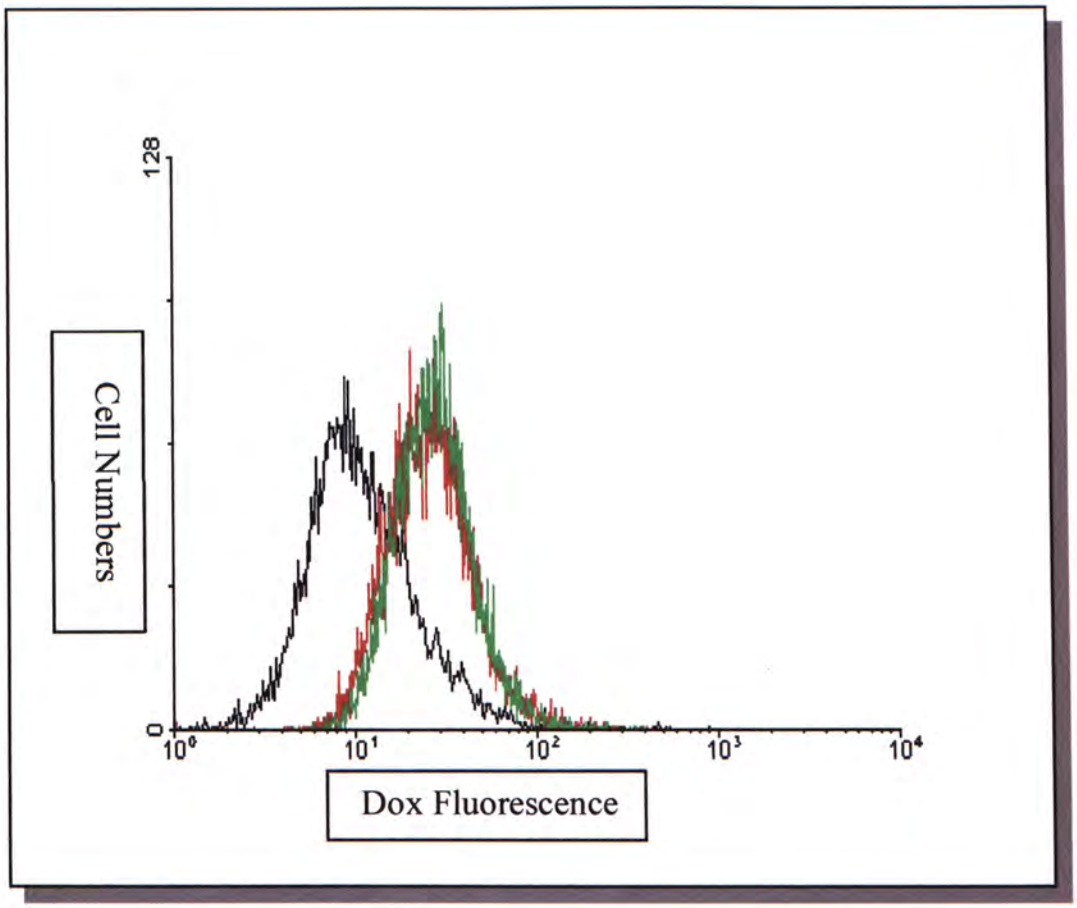
The results indicated that hyperthermia could enhance Dox accumulated in the R-HepG2 cells that was comparable to the percentage cells survival with and without the hyperthermia treatment. But hyperthermia could not increase the accumulation of LDL-Dox in the cells. Therefore, the hyperthermia

could enhance the Dox accumulation in the cells in order to increase the cytotoxic effect of Dox.



Sample	Mean Fluorescence Value / units
Control at 37°C	9.14
Dox at 37°C	12.52
Dox at 43°C	21.88

Fig. 3.33. The quantitative analysis of doxorubicin accumulation in R-HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6-well plate for 48 hours. Cells were treated with 1μM Dox for 1 hour at 37°C or 43°C. The accumulation of Dox was measured by flow cytometric analysis. The black line represented the control cells, i.e. cells incubated without Dox at 37°C. The red line represented the cells incubated with 1μM Dox at 37°C. The green line represented the cells incubated with 1μM LDL-Dox at 43°C.



Sample	Mean Fluorescence Value / units
Control at 37°C	9.14
LDL-Dox at 37°C	27.13
LDL-Dox at 43°C	27.59

Fig. 3.34. The quantitative analysis of low density lipoprotein-doxorubicin accumulated in R-HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6-well plate for 48 hours. Cells were treated with 1μM LDL-Dox for 1 hour at 37°C or 43°C. The accumulation of LDL-Dox was measured by flow cytometric analysis. The black line represented the control cells, i.e. cells incubated without LDL-Dox at 37°C. The red line represented the cells incubated with 1μM LDL-Dox at 37°C. The green line represented the cells incubated with 1μM LDL-Dox at 43°C.

3.1.3.7.Confocal laser scanning microscopic (CLSM) studies on the accumulation of Dox and LDL-Dox in R-HepG2 cells with and without hyperthermia

According to the result of flow cytometric analysis, when the R-HepG2 cells incubated with Dox at 43°C, the accumulation level of Dox in the cells was higher than Dox incubated at 37°C. When comparing the level of Dox accumulated in the cells incubated between 37°C and 43°C, it showed that the fluorescent intensity of Dox incubated at 43°C was higher than that incubated at 37°C. This result suggested that the intracellular level of Dox was enhanced by hyperthermia (Fig. 3.35).

In addition, from the result of the confocal laser scanning microscopic images of LDL-Dox-treated cells at 37°C and 43°C, it showed that the fluorescent intensity of LDL-Dox incubated at 37°C was similar to that at 43°C. That means there was no significant difference between applying hyperthermia or not (Fig. 3.36).

These results suggested that the combined treatment of Dox and hyperthermia could increase the intracellular level of Dox, but the intracellular level of LDL-Dox could not be enhanced by the hyperthermia and this result was comparable to the result of flow cytometric analyses.

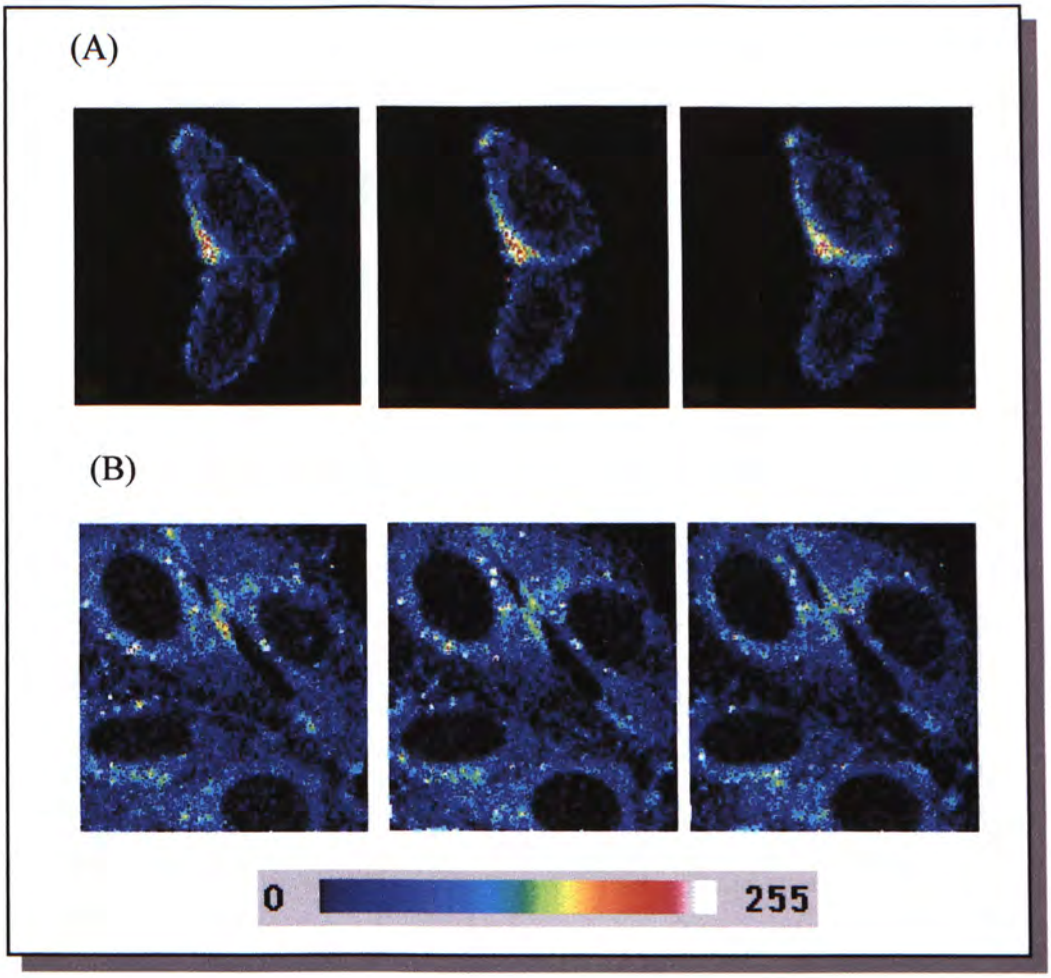


Fig. 3.35. The confocal laser scanning microscopic analysis on the accumulated level of Dox in R-HepG2 cells. Cell at 5 x 10^4 cells were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM Dox for 1 hour at 37°C or 43°C. (A), Dox-treated R-HepG2 cells incubated at 37°C were scanned along the Z-axis for 1μm. (B), Dox-treated R-HepG2 cells incubated at 43°C were scanned along Z-axis for 1μm.

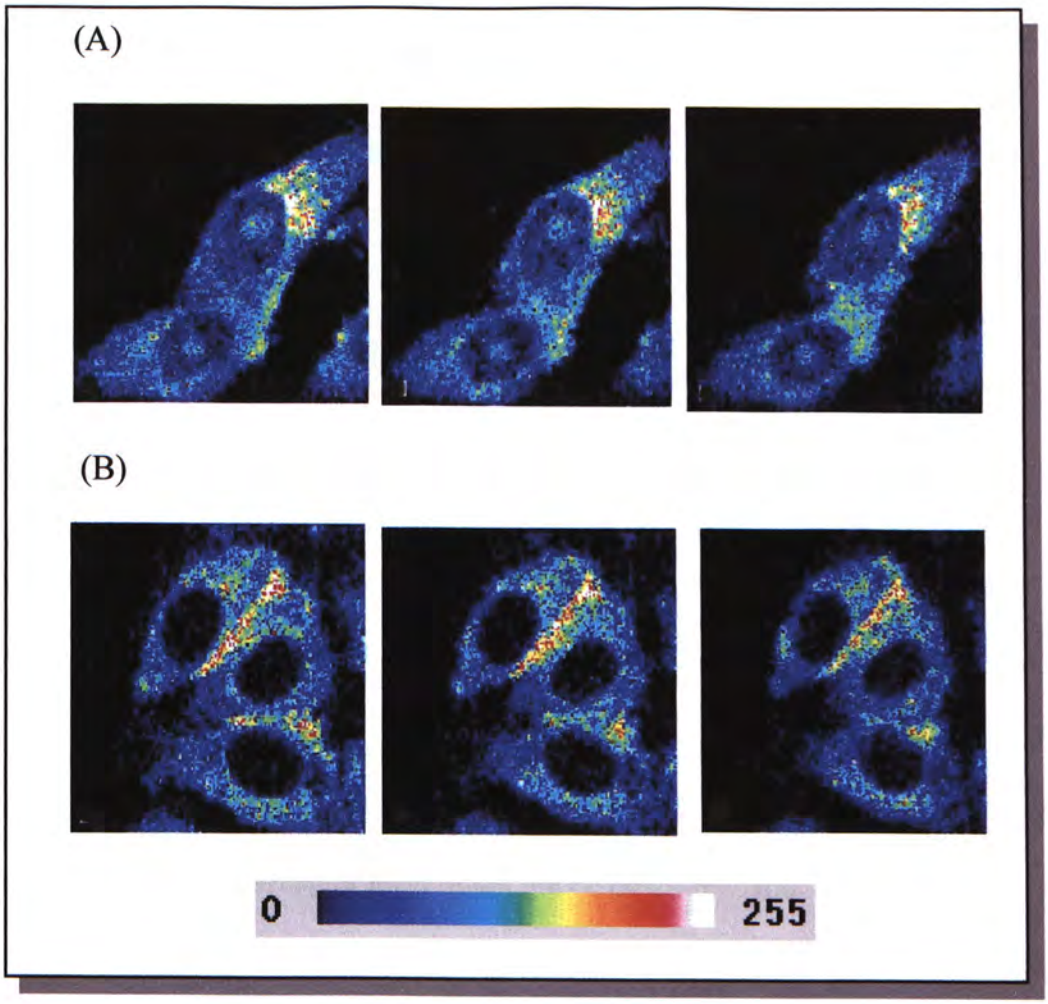


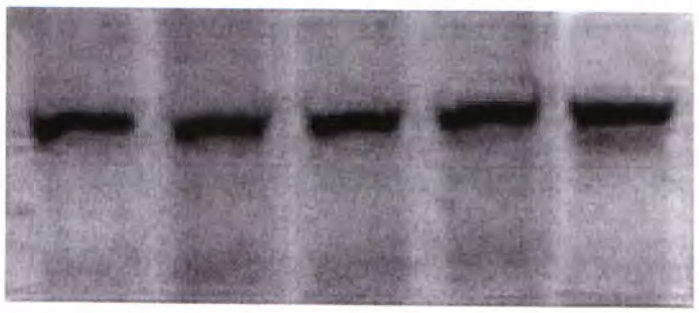
Fig. 3.36. The confocal laser scanning microscopic analysis on the accumulated level of LDL-Dox in R-HepG2 cells. Cells at 5 x 10^4 cells were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM LDL-Dox for 1 hour at 37°C or 43°C. (A), LDL-Dox-treated R-HepG2 cells incubated at 37°C were scanned along the Z-axis for 1μm. (B), LDL-Dox-treated R-HepG2 cells incubated at 43°C were scanned along Z-axis for 1μm.

3.1.3.8.Modulation of LDL receptors on R-HepG2 cells ------ Up-regulation of LDL receptors by *Fructus Crataegus* (FC)

3.1.3.8.1.The comparsion of LDL receptor expression on R-HepG2 cells after *Fructus Crataegus* (FC) pre-treatment

In R-HepG2 cells, when the cells treated with lipoprotein deficient serum (LPDS), the LDL receptor expression level was similar to that when the cells were treated with complete medium (control). Also the level of LDL receptor expression did not exhibit a very clear difference in cells between low density lipoprotein (LDL) treatment and LPDS treatment. This result showed that in R-HepG2 cells, the LDL receptor level might not be down-regulated by the existence of LDL. As the cells under *Fructus Crataegus* (FC) treatment, the receptor expression level was also similar to that when the cells were under LPDS treatment. That means the FC could not up-regulate LDL receptor expression in R-HepG2 cells. When the cells co-treated with FC and LDL, the expression level was nearly equal to that of the cells treated with FC and LDL only. Therefore, there was no significant difference on the LDL receptor expression level among these treatments (Fig. 3.37).

The result suggested that after FC pre-incubation, the LDL receptors expression level did not have a significant increase in R-HepG2 cells. Moreover, the LDL receptors expression was not suppressed by LDL.



Lane 1: Complete medium (CTL)

2: FC (1mg/ml) in LPDS

3: FC (1mg/ml) and LDL (0.2mg/ml) in LPDS

4: LDL (0.2mg/ml) in LPDS

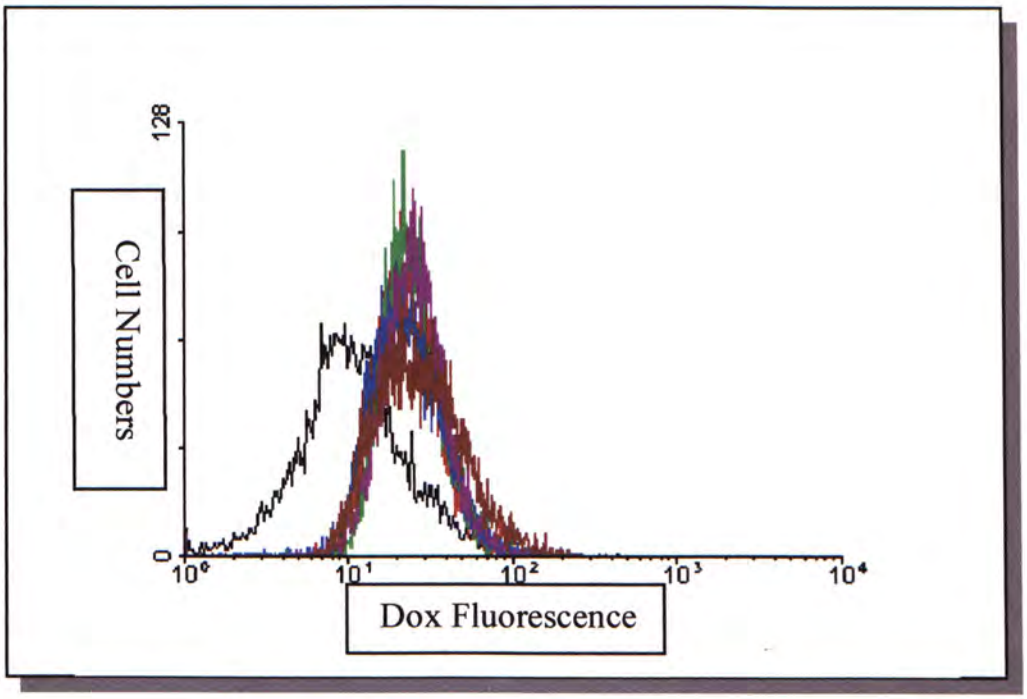
5: lipoprotein deficient serum (LPDS)

Fig.3.37. The expression level of LDL receptors in various treatments in R-HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6 well-plate overnight. Cells were treated with complete medium (CTL), lipoprotein deficient serum (LPDS), *Fructus Crataegus* (FC) in lipoprotein deficient serum, co-treatment of *Fructus Crataegus* and low density lipoprotein (FC and LDL) in lipoprotein deficient serum, low density lipoprotein (LDL) in lipoprotein deficient serum. The level of LDL receptor was analyzed by Western blot analysis. Aliquots of 25μg of protein were loaded in each lane.

3.1.3.8.2.The comparsion of the accumulation of LDL-Dox in R-HepG2 cells after *Fructus Crataegus* (FC) pre-treatment

As shown in Fig. 3.38, in R-HepG2 cells, the accumulation of LDL-Dox under complete medium (control) incubation was similar to that under LPDS incubation and the mean of the fluorescent intensity of cells was 21.10 and 22.27 respectively. There was no significant increase of LDL-Dox accumulation in cells between complete medium and LPDS treatment. After the cells were treated with LDL, the accumulation of LDL-Dox was similar to that of the cells after the LPDS treatment that means the existence of LDL was not a factor to affect the LDL-Dox bound to the LDL-receptors because there was only a little bit decrease of LDL-Dox accumulation when compared that in cells after LPDS treatment. After the cells were treated with *Fructus Crataegus* (FC), the LDL-Dox accumulation was also similar to that of the cells treated with LPDS and the mean of the fluorescent intensity of cells under FC-treatment was 23.08 in which there was no clear intensity difference when compared with that of cells after LPDS treatment, also mostly of the fluorescent intensity of cells after FC-treated was due to the Dox fluorescent, not due to the FC itself. That means that FC could not enhance the accumulation of LDL-Dox in R-HepG2 cells. As the cells were co-treated with FC and LDL, the LDL-Dox accumulation in the cells was nearly equal to that of the cells treated with FC. Thus the LDL-Dox accumulation in R-HepG2 cells was not affected by the pre-treatments.

These results suggested that the LDL-Dox accumulation in R-HepG2 cells was not significantly increased between various treatments. This phenomenon was comparable to the results in LDL receptor expression level under different kinds treatments as observed by Western blot analyses.



Sample	Mean Fluorescence Value / units
Control	9.82
Control + LDL-Dox	21.10
LPDS + LDL-Dox	22.27
LDL + LDL-Dox	21.48
FC + LDL-Dox	23.08
FC and LDL + LDL-Dox	22.07

Fig. 3.38. The quantitative analysis of LDL-Dox accumulation in R-HepG2 cells. Cells at 1 x 10^6 cells/well were seeded in 6-well plate overnight. Cells were treated with complete medium (control), lipoprotein deficient serum (LPDS), low density lipoprotein (LDL) in lipoprotein deficient serum, *Fructus Crataegus* (FC) in lipoprotein deficient serum and co-treatment of *Fructus Crataegus* and low density lipoprotein (FC and LDL) in lipoprotein deficient serum for 48 hours. After the pre-treatment, 1μM LDL-Dox mixing in lipoprotein deficient serum was added for 1 hour incubation. The accumulation of LDL-Dox was measured by flow cytometric analysis. The black line represented the control cells, which was not incubated with LDL-Dox. The red line represented the cells incubated with complete medium and 1μM LDL-Dox. The green line represented the cells incubated with lipoprotein deficient serum (LPDS) and 1μM LDL-Dox. The blue line represented the cells incubated with low density lipoprotein (LDL) and 1μM LDL-Dox. The purple line represented the cells incubated with *Fructus Crataegus* (FC) and 1μM LDL-Dox. The brown line represented the cells incubated with of *Fructus Crataegus* and low density lipoprotein (FC and LDL) and 1μM LDL-Dox.

3.1.3.8.3.Confocal laser scanning microscopic (CLSM) studies in the accumulation of LDL-Dox by *Fructus Crataegus* pre-treatment in R-HepG2 cells

In R-HepG2 cells, the confocal laser scanning microscopic images of LPDS-treated cells and complete medium-treated cells showed similar fluorescent intensity in the cells. This result suggested that the intracellular level of LDL-Dox under incubations of LPDS and complete medium was similar. After the cells were treated with LDL, there was not a clear difference of fluorescent intensity when comparing to the LPDS-treated cells, that means the existing LDL could not affect the intracellular level of LDL-Dox. When the cells were treated with FC, the fluorescent intensity of cells was similar to that of the cells treated with LPDS, i.e. the intracellular level of LDL-Dox could not be increased by FC pre-treatment and most of the fluorescent intensity of cells was due to the Dox fluorescent, but not due to the fluorescent of FC itself. As the cells were co-treated with FC and LDL, there was no significant difference in the fluorescent intensity of cells between FC and LDL treated only (Fig. 3.39 and Fig. 3.40) This phenomenon was comparable to the results in the LDL-Dox accumulation in the cells under different kinds of the treatments obtained by flow cytometric analysis.

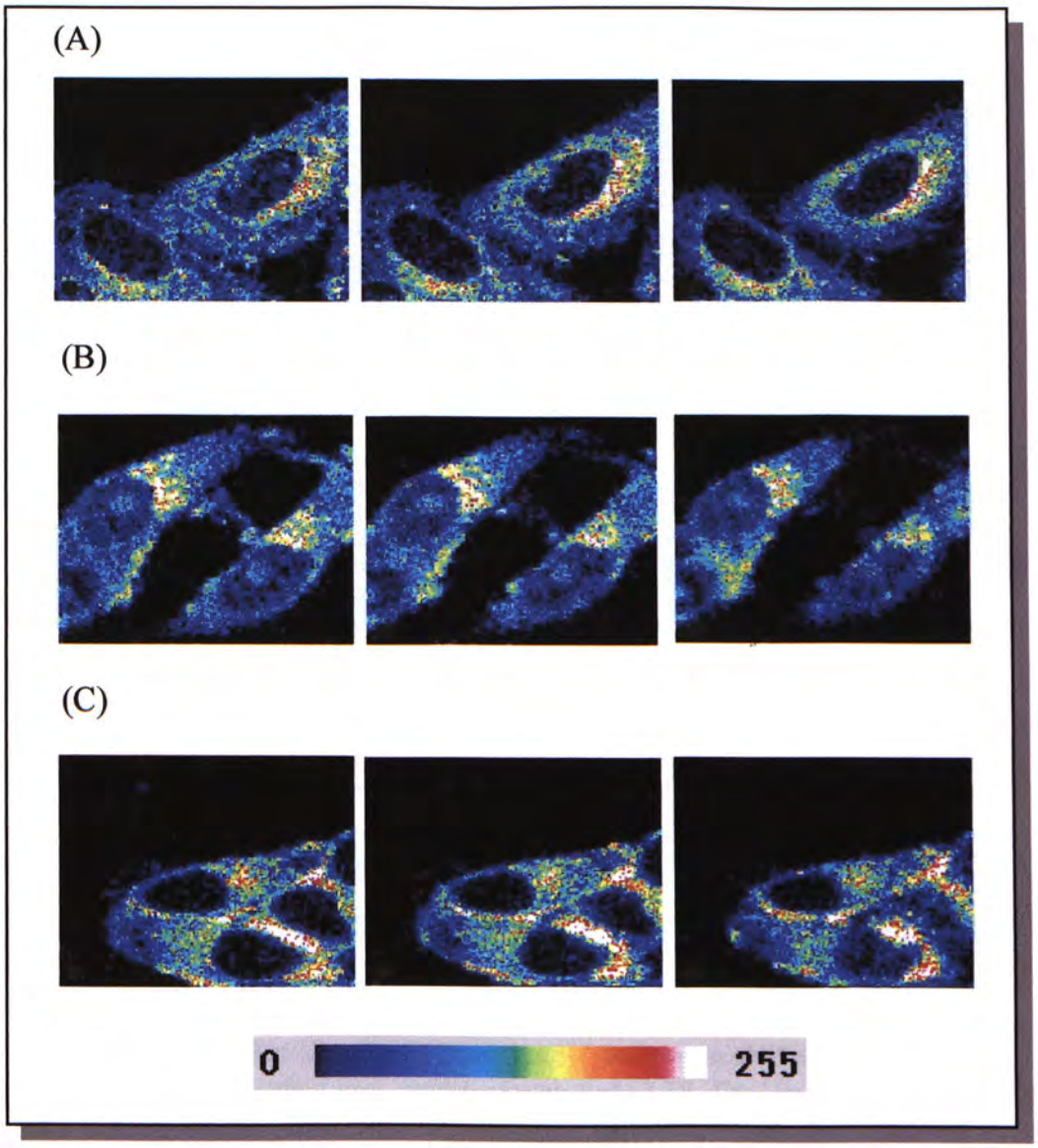


Fig. 3.39 The confocal laser scanning microscopic analysis on the accumulated level of LDL-Dox in R-HepG2 cells. Cells at 5 x 10^4 cells were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM LDL-Dox for 1 hour at 37°C. (A), complete medium (control)-treated R-HepG2 cells were scanned along the Z-axis for 1μm. (B), lipoprotein deficient serum (LPDS)-treated R-HepG2 cells were scanned along Z-axis for 1μm. (C), *Fructus Crataegus* (FC) in LPDS-treated R-HepG2 cells were scanned along Z-axis for 1μm.

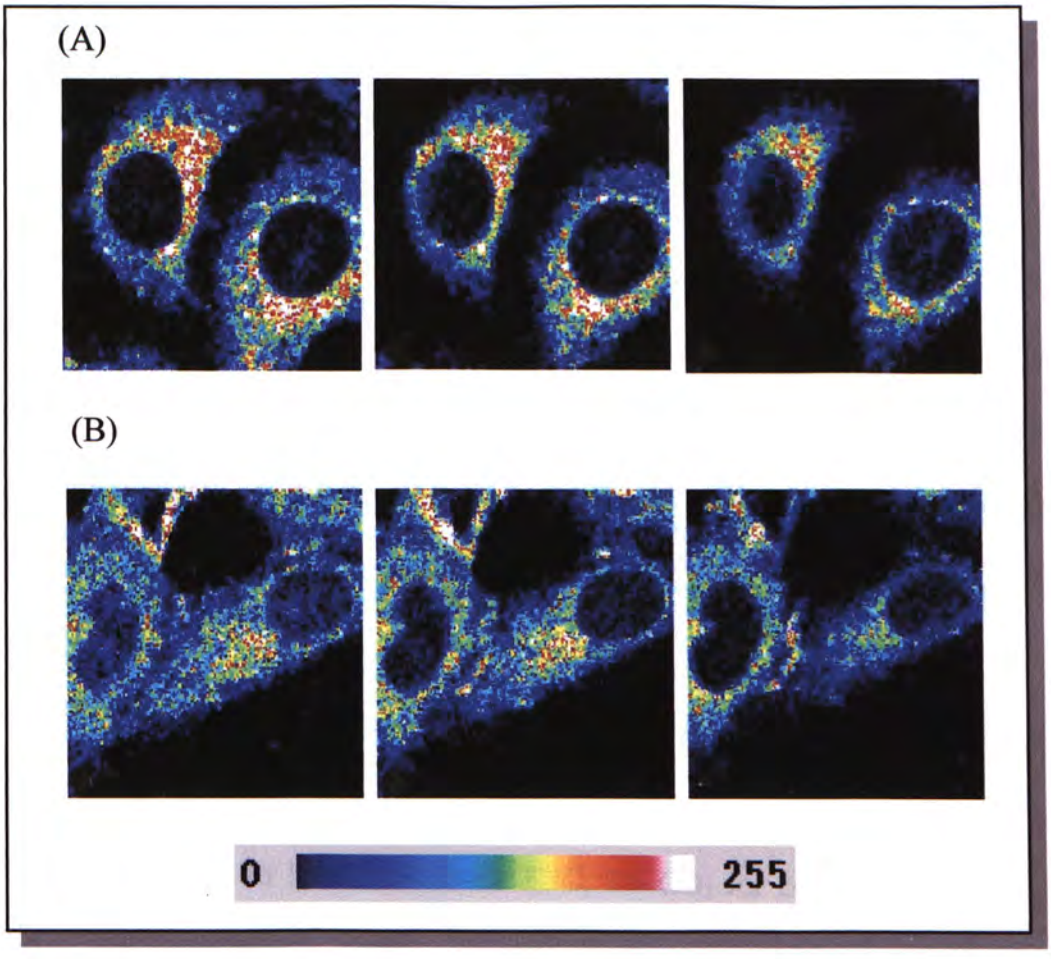


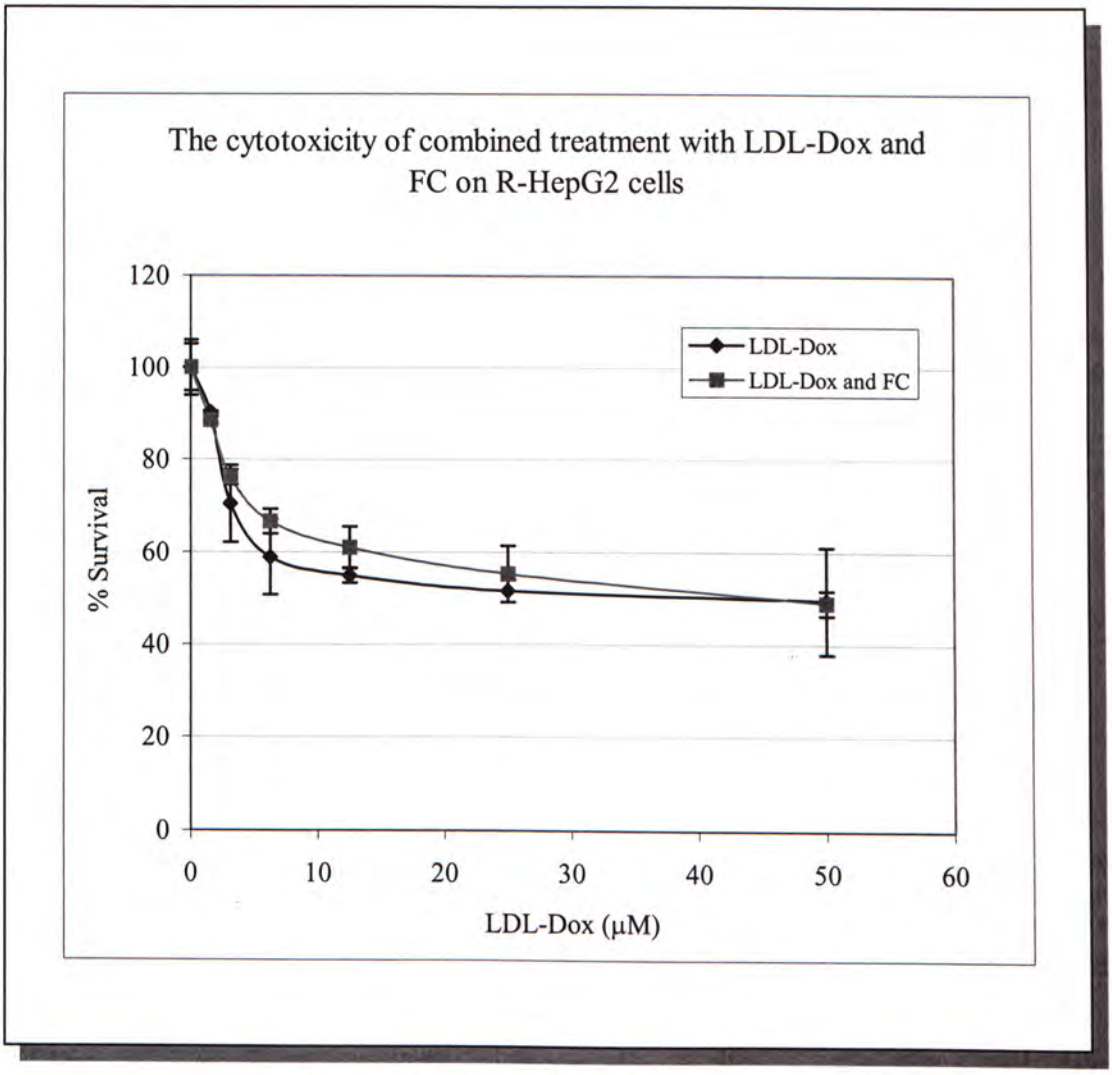
Fig. 3.40. The confocal laser scanning microscopic analysis on the accumulated level of LDL-Dox in R-HepG2 cells. Cells at 5 x 10^4 cells were seeded on cover glass for 48 hours. The cover glass was incubated with 5μM LDL-Dox in LPDS for 1 hour at 37°C. (A), co-treaed with *Fructus Crataegus* and low density liporotein (FC and LDL) in LPDS on R-HepG2 cells were scanned along the Z-axis for 1μm. (B), low density lipoprotein in LPDS treated in R-HepG2 cells were scanned along Z-axis for 1μm.

3.1.3.8.4.The comparison of cytotoxicity of combined treatment with LDL-Dox and *Fructus Crataegus* (FC) in R-HepG2 cells

After the cells were treated with FC, the results showed that the protein expression level of LDL receptors and the accumulation of LDL-Dox in the cells did not exhibit a significant increase. So theoretically, the percentage of cell survival under the combined treatment of FC and LDL-Dox should be similar to that of the cells under the LDL-Dox treatment only.

The R-HepG2 cells were pre-treated with FC in LPDS medium up to 48 hours, then the cells were incubated at different concentrations of LDL-Dox ranging from 50μM to 1.5626μM under 37°C, 5% CO_2 for 24 hours and the cells viability was determined by MTT assay. After the cells pre-treated with FC, the IC_{50} of LDL-Dox was 47.8μM which was similar to that of the LDL-Dox treatment only, and the IC_{50} of LDL-Dox after 24 hours treatment without FC-treated was 48μM (Fig. 3.41). Moreover, there was not any cytotoxic effect of 1mg/ml FC on HepG2 cells observed in the previous studies, thus the cytotoxicity of R-HepG2 cells determined in this assay was due to the cytotoxic effect of LDL-Dox.

These results suggested that after FC pre-treatment, the LDL receptor expression level did not exhibit a significant increase, so the LDL-Dox accumulation in the cells was not increased and the cytotoxic effect of LDL-Dox was not enhanced by the FC pre-treatment.



Sample	The value of IC_{50}
LDL-Dox only	48
LDL-Dox and FC-treatment	47.8

Fig. 3.41. Effect of combined treatment with low density lipoprotein-doxorubicin and the pre-treatment of *Fructus Crataegus* (FC) on the survival of R-HepG2 cells. Cells at 5 x 10^3 cells/well were seeded in a 96 wells plate overnight. Cells were treated with 1mg/ml of FC in lipoprotein deficient serum for 48 hours. After 48 hours, the cells were washed with 1X PBS twice, then the cells were incubated at different concentration of LDL-Dox for further 24 hours. The percentage of cell survival was analyzed by MTT assay. In each well, 30μl MTT solution and 100μl DMSO were added and the absorbance of the mixture was measured at wavelength 540nm. The % survival of each incubation was calculated as described in Material & Methods.

3.2. *IN VIVO* STUDIES

3.2.1. The comparison of Dox and LDL-Dox on reducing the tumor sizes and weight in nude mice bearing R-HepG2 cells

The effect of LDL-Dox on inhibiting the tumors was also investigated by measuring the tumor size and tumor weight when the mice were sacrificed.

3.2.1.1.The comparison of Dox and LDL-Dox on reducing the tumor size in nude mice bearing R-HepG2 cells

As shown in Fig. 3.42, after 4 weeks treatment, the tumor size of 1mg/kg Dox treated group was similar to that of the control group and there was no significant difference between the two groups ($p>0.5$). But the mice treated with 2mg/kg Dox showed a smaller tumor size when compared with that of the control group ($p<0.001$) and 1mg/kg Dox treated group ($p<0.005$). Also, the LDL-Dox treated group had a smaller size compared with that of the control group and 1mg/kg Dox treated group ($p<0.001$). But there was no significant difference between 2mg/kg Dox treated group and 1mg/kg LDL-Dox treated group ($p>0.5$). This result shows that the antiproliferative effect of 1mg/kg LDL-Dox was greater than that of 1mg/kg Dox on R-HepG2 cells growing on the shoulder of the nude mice. Moreover, the antiproliferative effect of 1mg/kg LDL-Dox was similar to that of 2mg/kg Dox.

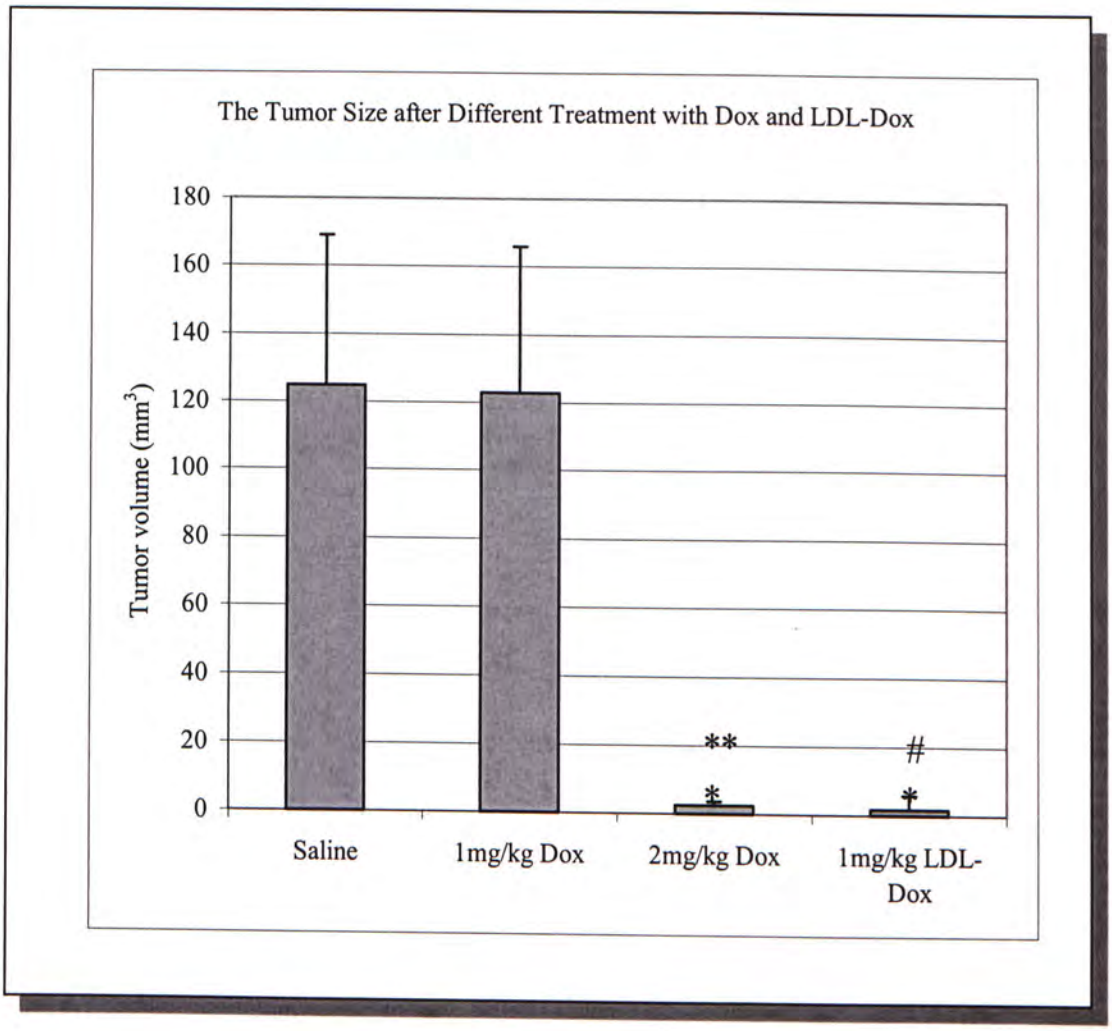


Fig. 3.42. Size of tumors after Dox and LDL-Dox treatment on R-HepG2 cells growing in nude mice. Nude mice were injected subcutaneously with 8 x 10^6 of human resistance hepatoma R-HepG2 cells in the shoulder. After 2 days of inoculation, LDL-Dox and Dox at a dose of 1mg/kg as well as Dox at a dose of 2mg/kg was injected intravenously on every other day. The sizes of tumor were measured after the end of the treatment. (*: $p<0.001$ compared with control group, **: $P<0.005$ compared with 1mg/kg Dox treated group, # : $P<0.001$ compared with 1mg/kg Dox treated group)

After 4 weeks treatment, the tumor sizes of R-HepG2 cells on nude mice under different treatments were shown at Figure 3.43, Figure 3.44, Figure 3.45, Figure 3.46 and Figure 3.47.

Fig 3.43. Normal nude mice without receiving any injection of human resistant hepatoma R-HepG2 cells.

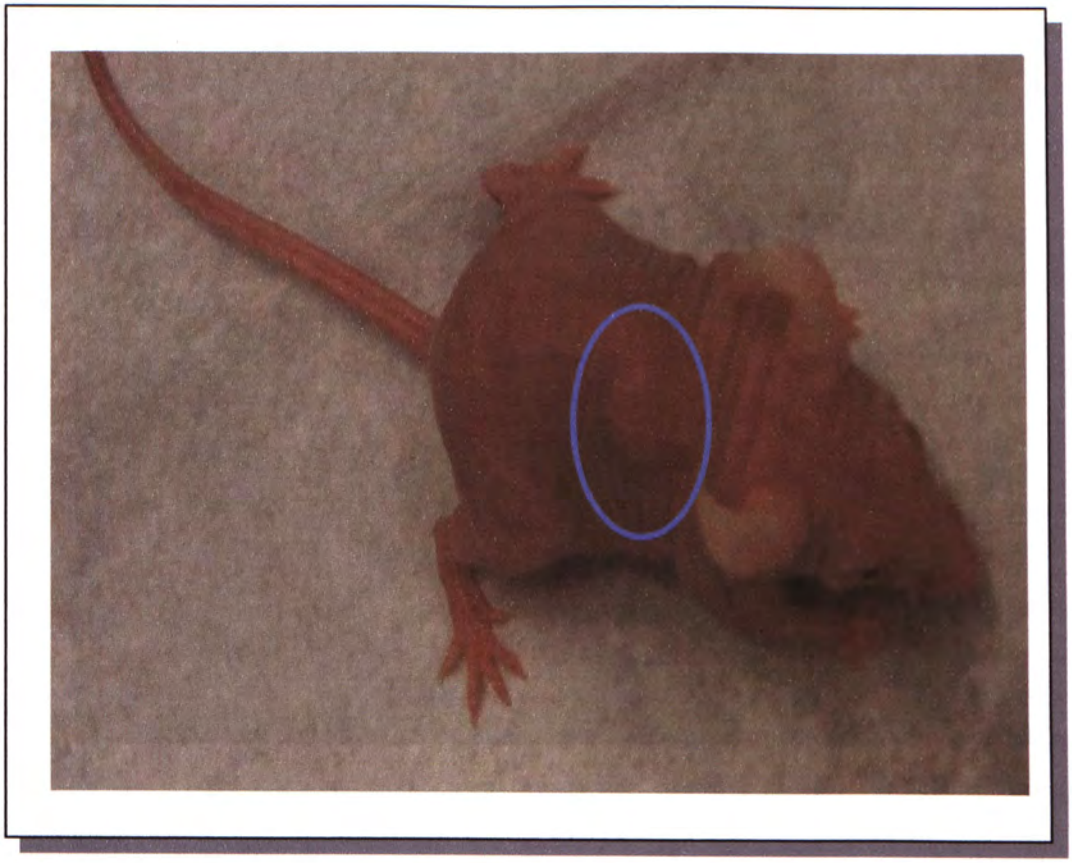


Fig. 3.44. Size of tumors of nude mice bearing R-HepG2 cells treated with saline only. Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After 2 days of inoculation, saline was injected intravenously on every other day until 4 weeks.

Fig. 3.45. Size of tumors of nude mice bearing R-HepG2 cells treated with 1mg/kg Dox. Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After 2 days of inoculation, Dox at a dose of 1mg/kg was injected intravenously on every other day until 4 weeks.

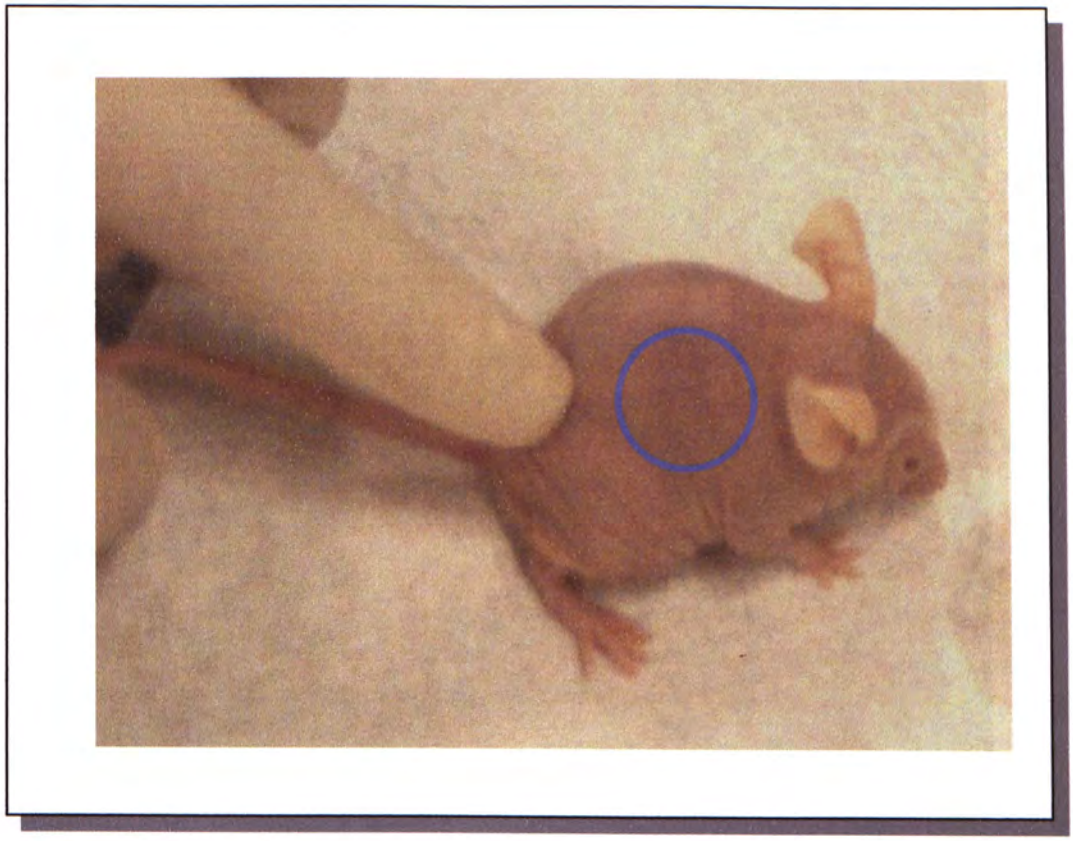


Fig. 3.46. Size of tumors of nude mice bearing R-HepG2 cells treated with 2mg/kg Dox. Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After 2 days of inoculation, Dox at a dose of 2mg/kg was injected intravenously on every other day until 4 weeks.

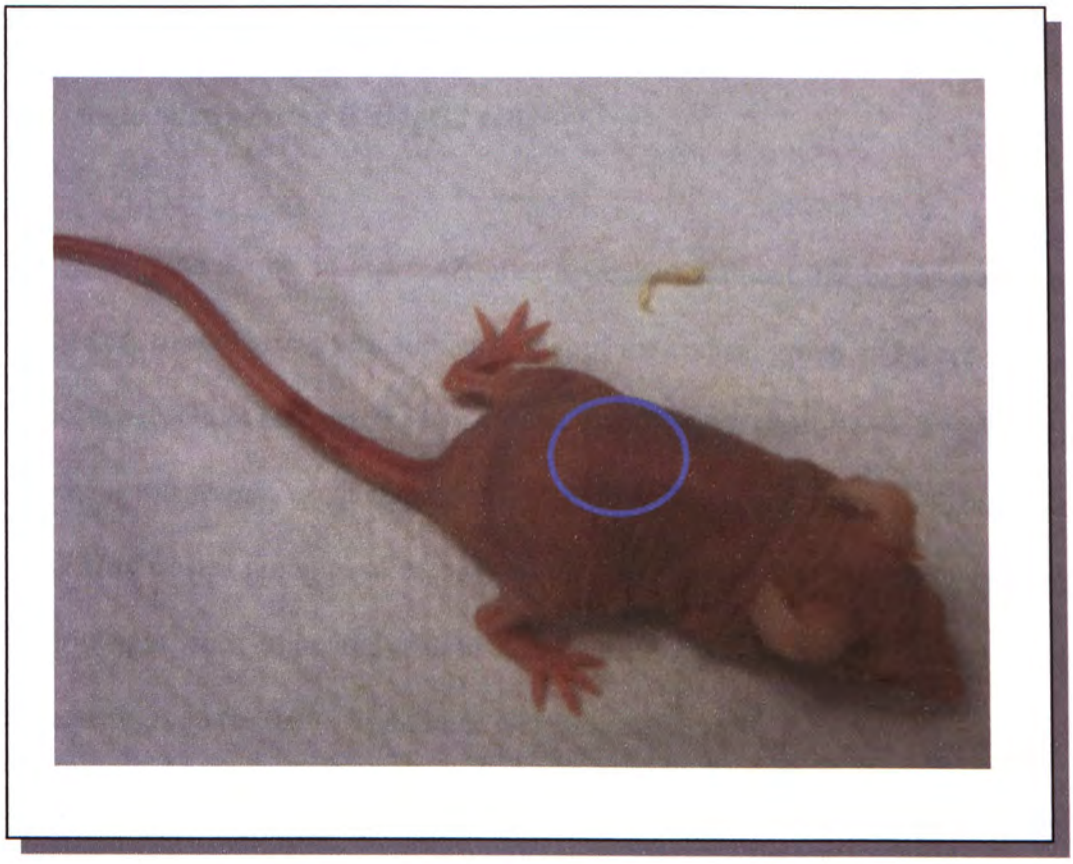


Fig. 3.47. Size of tumors of nude mice bearing R-HepG2 cells treated with 1mg/kg LDL-Dox. Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After 2 days of inoculation, LDL-Dox at a dose of 1mg/kg was injected intravenously on every other day until 4 weeks.

3.2.1.2.The comparison of Dox and LDL-Dox on reducing the tumor weight in nude mice bearing R-HepG2 cells

As shown in Fig. 3.48, after 4 weeks treatment, the tumor weight of 1mg/kg Dox treated group was similar to that of the control group and there was no significance different between the two groups. But the mice treated with 2mg/kg Dox showed a lower weight when compared with that of the control group ($p<0.005$) and 1mg/kg Dox treated group ($p<0.05$). Moreover, 1mg/kg LDL-Dox treated group had a lower weight when compared with that of the control group ($p<0.005$) and 1mg/kg Dox treated group ($p<0.05$). The tumor weight under 2mg/kg Dox treated group and 1mg/kg LDL-Dox treated group was similar. This result suggested that the antiproliferative effect of 1mg/kg LDL-Dox and 2mg/kg Dox was greater than that of 1mg/kg Dox on R-HepG2 cells growing on the shoulder of the nude mice. Furthermore, the antiproliferative effect of 1mg/kg LDL-Dox and 2mg/kg Dox was similar.

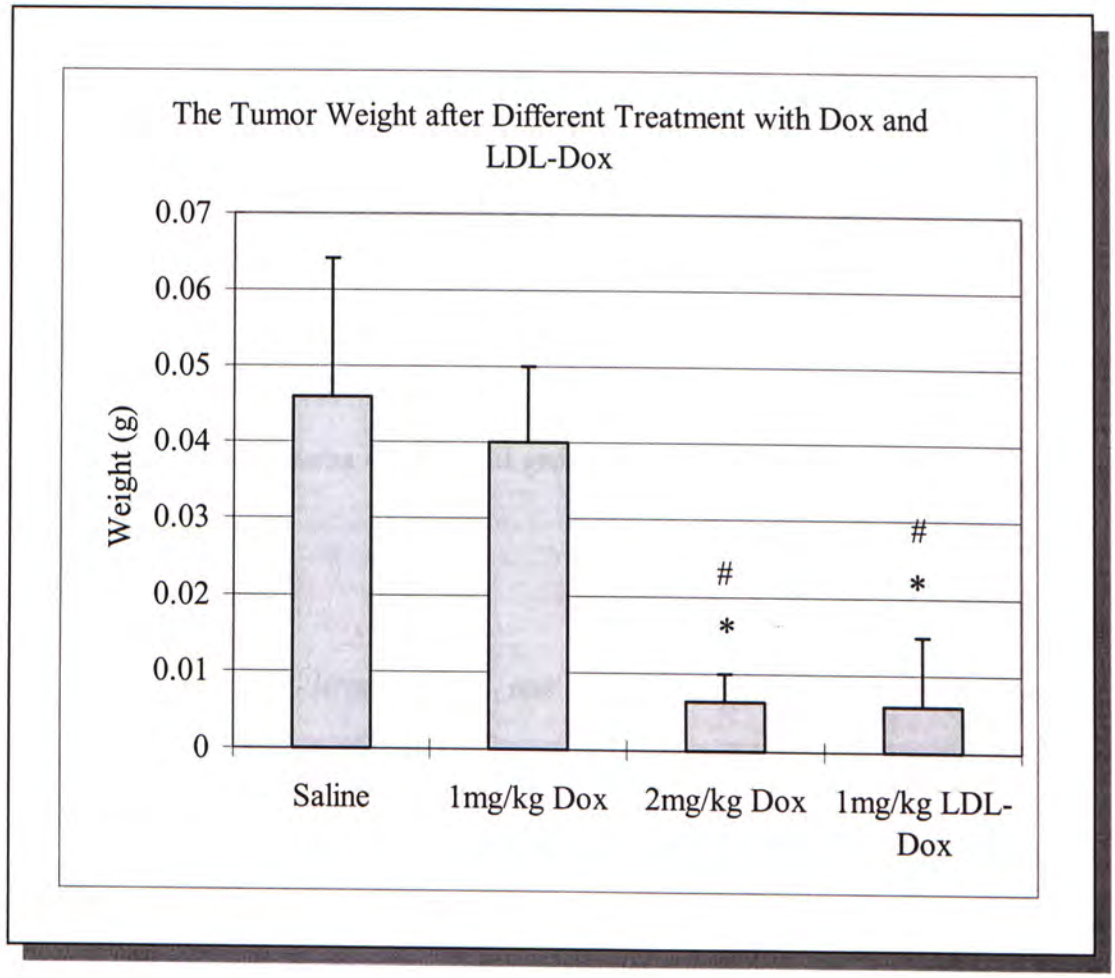


Fig. 3.48. Weight of tumors of nude mice bearing R-HepG2 cells treated with Dox and LDL-Dox. Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After 2 days of inoculation, LDL-Dox and Dox at a dose of 1mg/kg and Dox and at dose of 2mg/kg Dox was injected intravenously on every other day. After 4 weeks treatment, tumors were removed on these ether anesthetized mice and weighted. (*: $p<0.005$ compared with control group, #: $p<0.05$ compared with 1mg/kg Dox-treated group)

3.2.2. Myocardial injury measured by Lactate dehydrogenase (LDH) activity in nude mice bearing R-HepG2 cells treated with Dox and LDL-Dox

Lactate dehydrogenase (LDH) catalyzes the interconversion of lactate and pyruvate. LDH plasma activity in plasma is significantly elevated during myocardial injury. Plasma of different groups of mice was collected as described in the Methods section.

In the investigation, one group of nude mice did not receive subcutaneous injection of the R-HepG2 cells was called "none group". As shown in Fig. 3.49, the LDH plasma activity in control group, which was injected with saline, was similar to that of the none group and the mean of LDH activity in none group and the control group were 37.47 U/L and 41.30 U/L respectively, without significant difference. Moreover, the LDH plasma activity was higher in the Dox-treated group when compared with that of the control group after 4 weeks treatment ($p<0.05$). The mean of LDH plasma activity in 1mg/kg Dox-treated group was 89.13 U/L and 2mg/kg Dox-treated group was 99.85 U/L. Also, the LDH plasma activity in high dose of Dox treated group was higher than that in low dose of Dox treated group. On the other hand, LDH plasma activity in LDL-Dox treated group was similar to that in the control group. The mean LDH plasma activity in LDL-Dox treated group was 36.80 U/L while that for the control group was 41.30 U/L. Moreover, the LDH plasma activity in LDL-Dox treated group was lower than that in the Dox treated group ($p<0.05$).

This result indicated that no heart damage was observed in LDL-Dox treated group. In addition, the level of heart damage in Dox-treated group was higher than that in LDL-Dox treated group. Also, the level of heart damage was dose-dependent of Dox, ie. the higher dosage of Dox used, the higher level of heart damage could be observed.

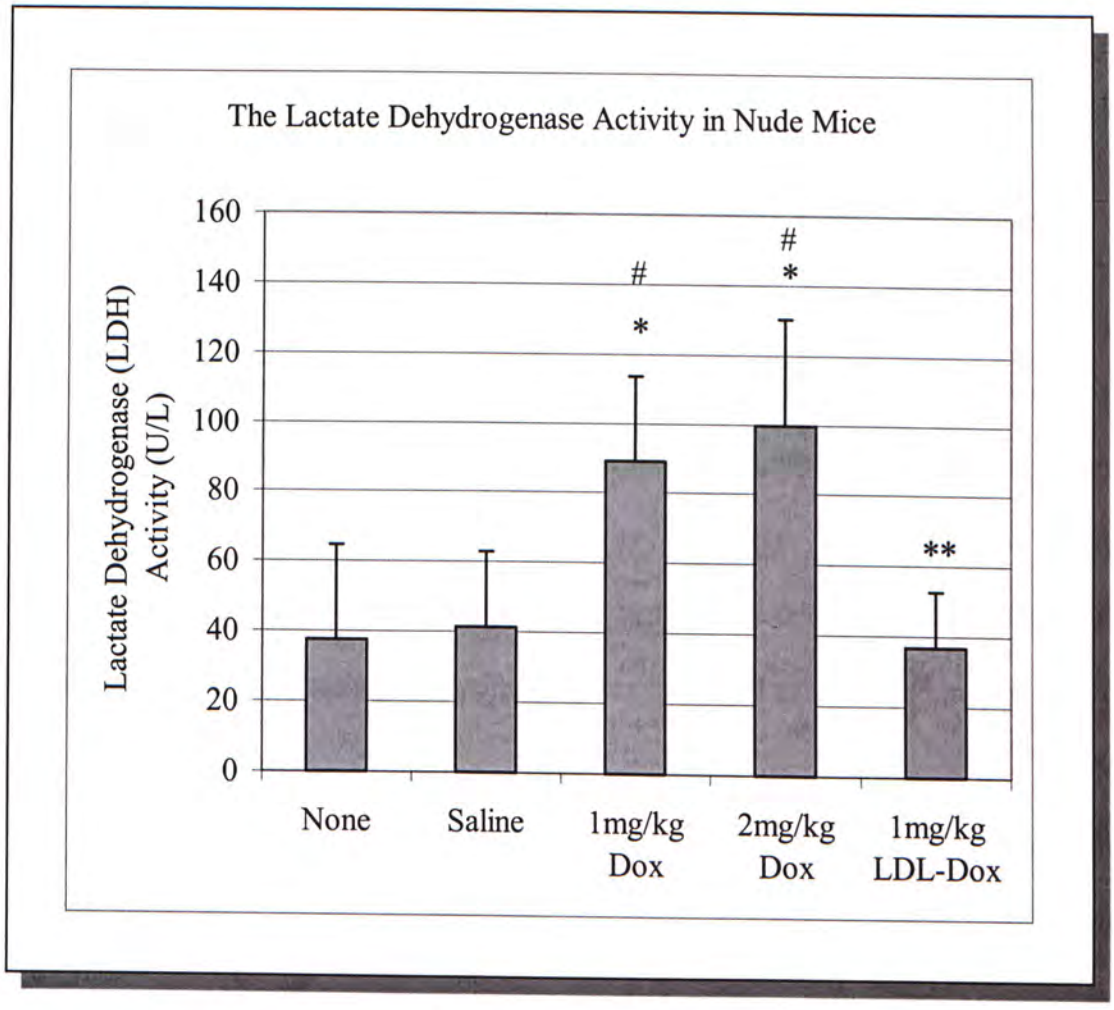


Fig. 3.49. Cardiotoxic effects of Dox and LDL-Dox in nude mice bearing R-HepG2 cells. Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After 2 days of inoculation, LDL-Dox and Dox at a dose of 1mg/kg and Dox at dose 2mg/kg was injected intravenously on every other day. After 4 weeks treatment, a heparinized blood sample was collected from ether anesthetized mice by cardiac puncture. The blood was then centrifuged at 1000x g for 15 minutes. The plasma obtained from the upper clear fraction was then analyzed for the LDH plasma activity. (*: $p<0.05$ compared with none group, #: $p<0.05$ compared with control group, **: $p<0.05$ compared with 1mg/kg Dox treated group and 2mg/kg Dox treated group)

3.2.3. Myocardial injury measured by Creatine kinase (CK) activity in nude mice bearing R-HepG2 cells treated with Dox and LDL-Dox

Creatine kinase (CK) is primarily located in skeletal muscle, brain tissue and heart muscle. Damage to these tissues results in the release of increased levels of creatine kinase into blood. Cardiac muscle injury following myocardial infarction results in a rise in serum CK activity. Therefore, measurement of serum CK activity is one of the useful diagnostic markers to detect myocardial injury. Plasma samples of different groups of mice were collected as described in Methods.

There was no significant difference between the none group and the control group in CK plasma activity. The CK plasma activity was higher in the Dox-treated group when compared with that of the control group after 4 weeks treatment. The mean CK plasma activity of 1mg/kg Dox-treated group and 2mg/kg Dox-treated group were 485.77 U/L and 496.37 U/L respectively. On the other hand, CK plasma activity in LDL-Dox treated group was similar to that of the control group. The mean CK plasma activity in LDL-Dox treated group was 396.20 U/L while that for the control group was 394.69 U/L. Moreover, the CK plasma activity in LDL-Dox treated group was lower than that in Dox-treated group (Fig. 3.49).

This result indicates that no heart damage was observed in LDL-Dox treated group. In addition, the heart damage in Dox-treated group was higher than that in LDL-Dox treated group. The level of heart damage was dose-dependent, ie.

the higher concentration of Dox used, the higher level of heart damage could be observed.

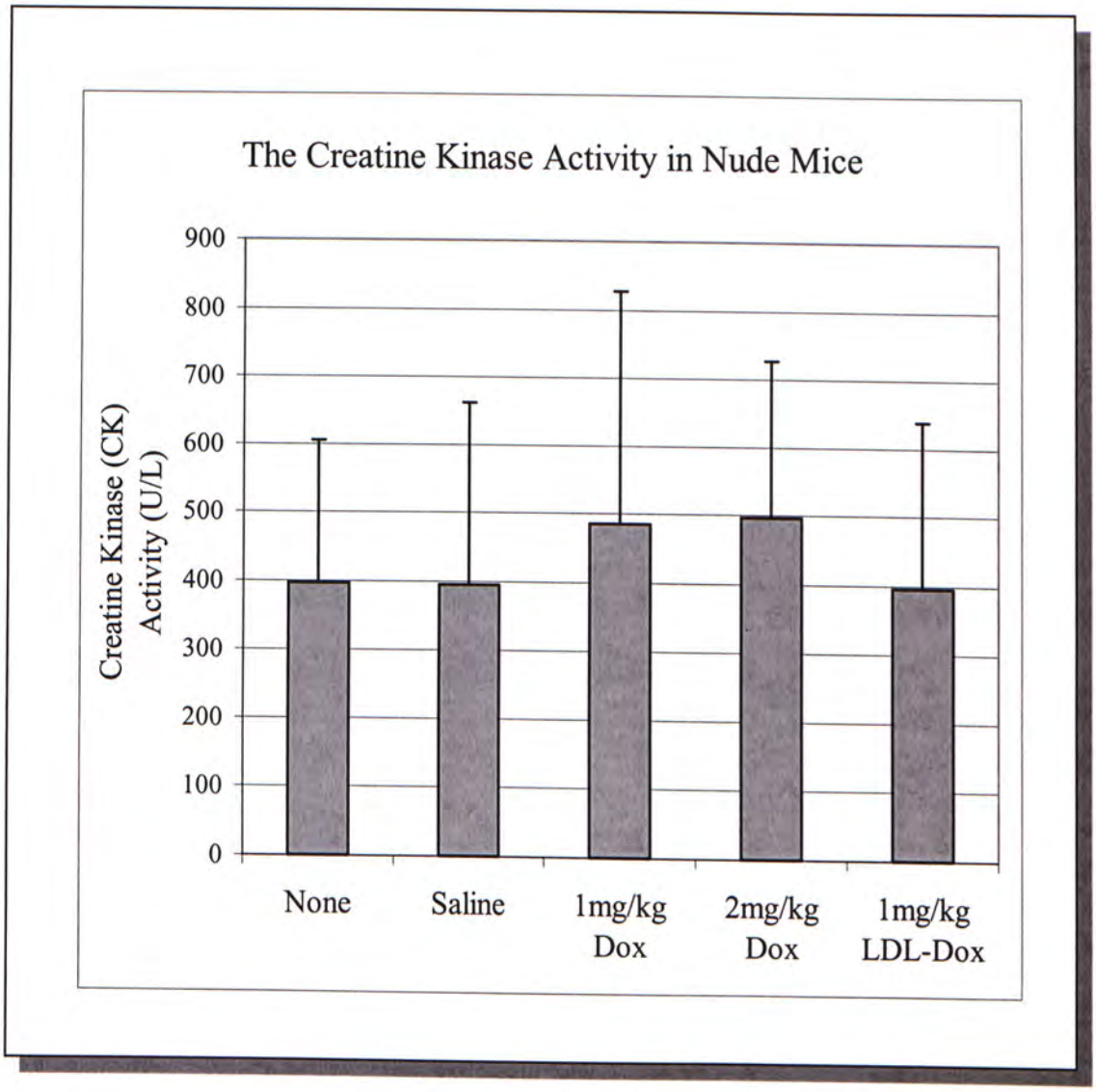


Fig. 3.49. Cardiotoxic effects of Dox and LDL-Dox in nude mice bearing R-HepG2 cells. Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After 2 days of inoculation, LDL-Dox and Dox at a dose of 1mg/kg and Dox at a dose of 2mg/kg was injected intravenously on every other day. After 4 weeks treatment, a heparinized blood sample was collected from ether anesthetized mice by cardiac puncture. The blood was then centrifuged at 1000x g for 15 minutes. The plasma obtained from the upper clear fraction was then analyzed for the CK plasma activity.

3.2.4. Histological studies of heart of nude mice bearing R-HepG2 cells treated with Dox and LDL-Dox

3.2.4.1.Heart section of nude mice

The heart section from nude mice was observed under light microscope. In this group (none group), the nude mice did not receive any R-HepG2. In this heart section of none group, the pattern of mycocardial muscle alignment and the location of muscle cell nuclei could be observed. It was shown that the mycocardial filament were well organized, smooth and tightly packed (Fig. 3.50).

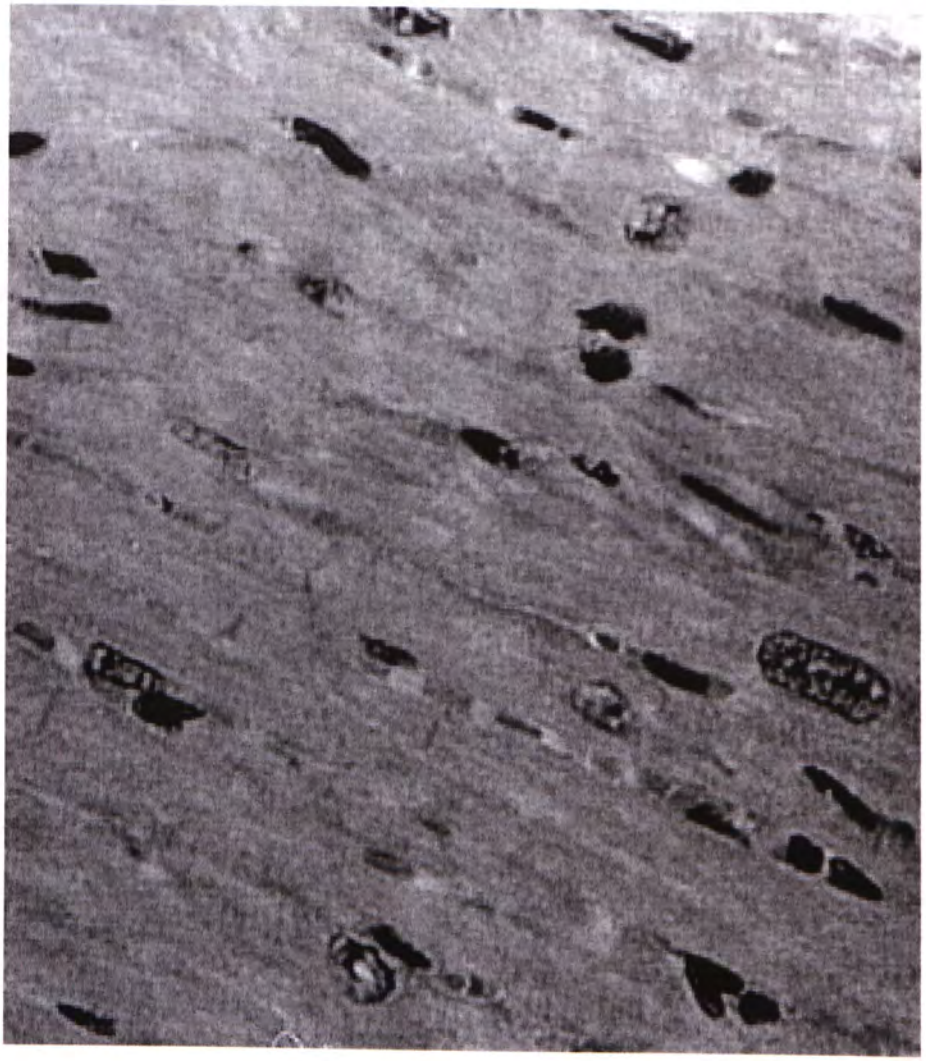


Fig. 3.50. Heart section of nude mice without subcutaneously of R-HepG2 cells (None group)
Magnification: 2.5 x 40 x 1 x 4 = 400x
Sample: ventricular wall portion
Nude mice did not receive any human resistant hepatoma R-HepG2 cells. During the treatment, the mice did not have any injection. After 4 weeks treatment, the mice were sacrificed by cervical dislocation and the hearts were quickly removed for histological slide preparation.

3.2.4.2.Heart section of nude mice bearing R-HepG2 cells

The heart section from the tumor bearing nude mice was observed under light microscope. It was obtained from the control group in which no Dox and LDL-Dox was injected to the mice but only saline was injected. In this heart section of control group, the pattern of myocardial muscle alignment and the location of muscle cell nuclei could be observed. It was found that the myocardial filaments were well organized, smooth and tightly packed. Moreover, there was no clear difference in the pattern of mycocardial filaments between the none group and the control group (Fig. 3.51).

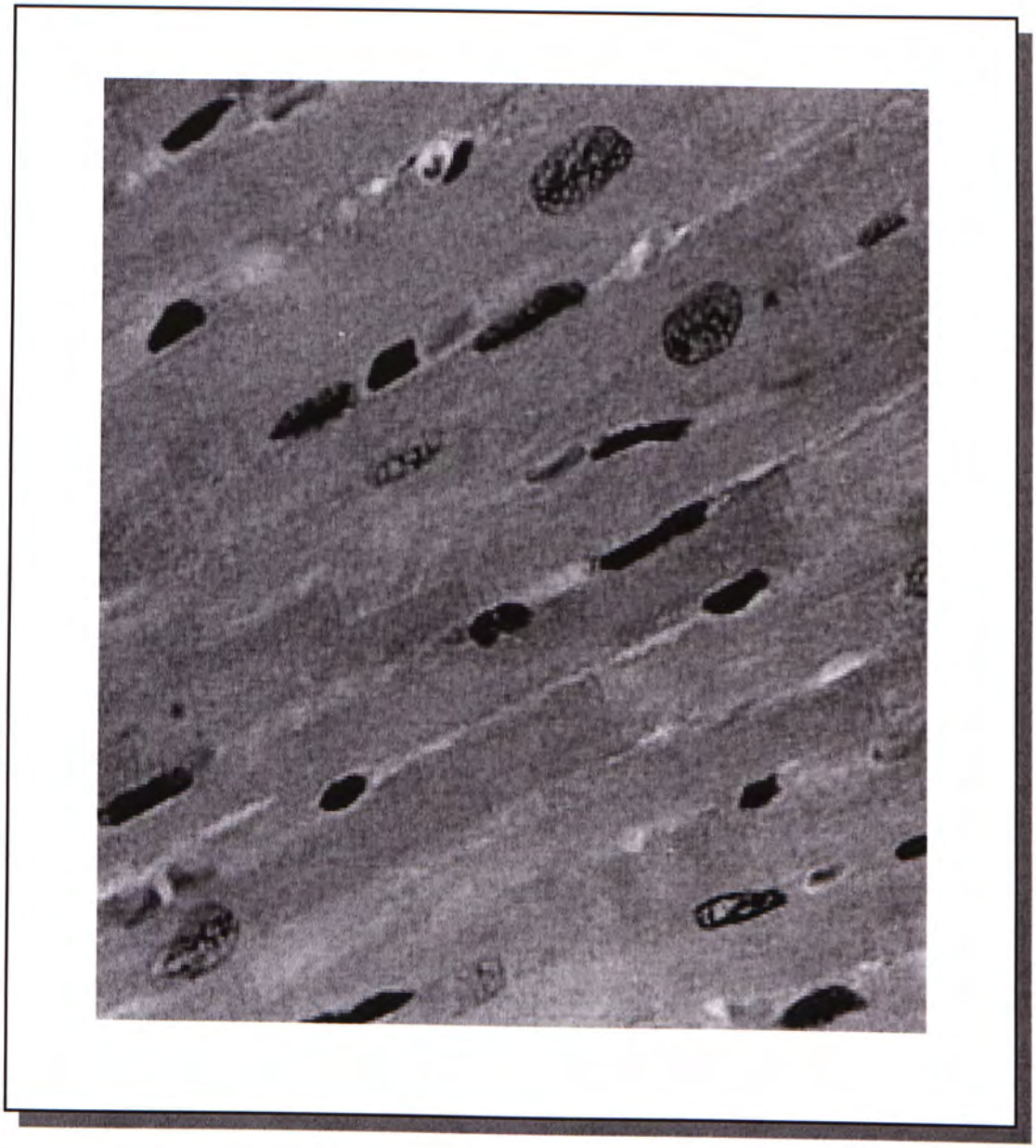


Fig. 3.51. Heart section of R-HepG2 bearing nude mice (Control group)
Magnification: 2.5 x 40 x 1 x 4 = 400x
Sample: ventricular wall portion
Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After two days of inoculation, saline was injected intravenously on every other day. After 4 weeks treatment, the mice were sacrificed by cervical dislocation and the hearts were quickly removed for histological slide preparation.

3.2.4.3.Heart section of 1mg/kg Dox treated nude mice bearing R-HepG2 cells

The heart section from the 1mg/kg Dox treated nude mice bearing R-HepG2 cells was observed under light microscope. Dox at a dose of 1mg/kg was injected to these mice intravenously on every other day. It was found that the organization of myocardial filaments was disrupted by Dox and vacuolization was found when compared with control group (Fig. 3.52).

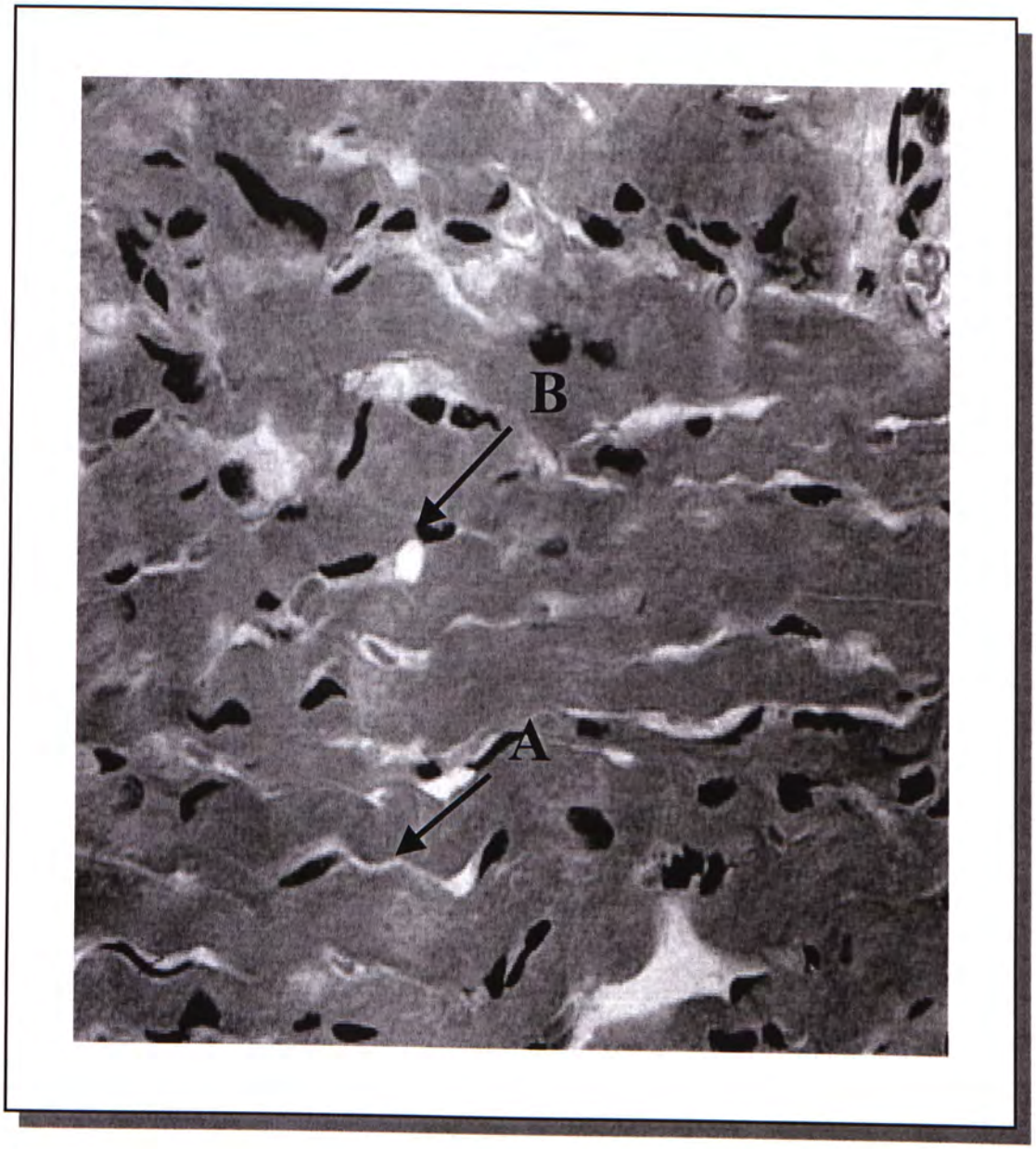


Fig. 3.52. Heart section of R-HepG2 bearing nude mice treated with 1mg/kg Dox
Magnification: 2.5 x 40 x 1 x 4 = 400x
Sample: ventricular wall portion
Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After 2 days of inoculation, Dox at a dose of 1mg/kg was injected intravenously on every other day. After 4 weeks treatment, the mice were sacrificed by cervical dislocation and the hearts were quickly removed for histological slide preparation. A: the disrupted myocardial filament; B: vancuolization.

3.2.4.4.Heart section of 2mg/kg Dox treated nude mice bearing R-HepG2 cells

The heart section from the 2mg/kg Dox treated nude mice bearing R-HepG2 cells was observed under light microscope. Dox at a dose of 2mg/kg was injected to these mice intravenously on every other day. It was found that the organization of myocardial filaments was disrupted by Dox and vacuolization was found when compared with control group. Moreover, the disruption of filament treated with 1mg/kg Dox was lower than that treated with 2mg/kg Dox (Fig. 3.53).

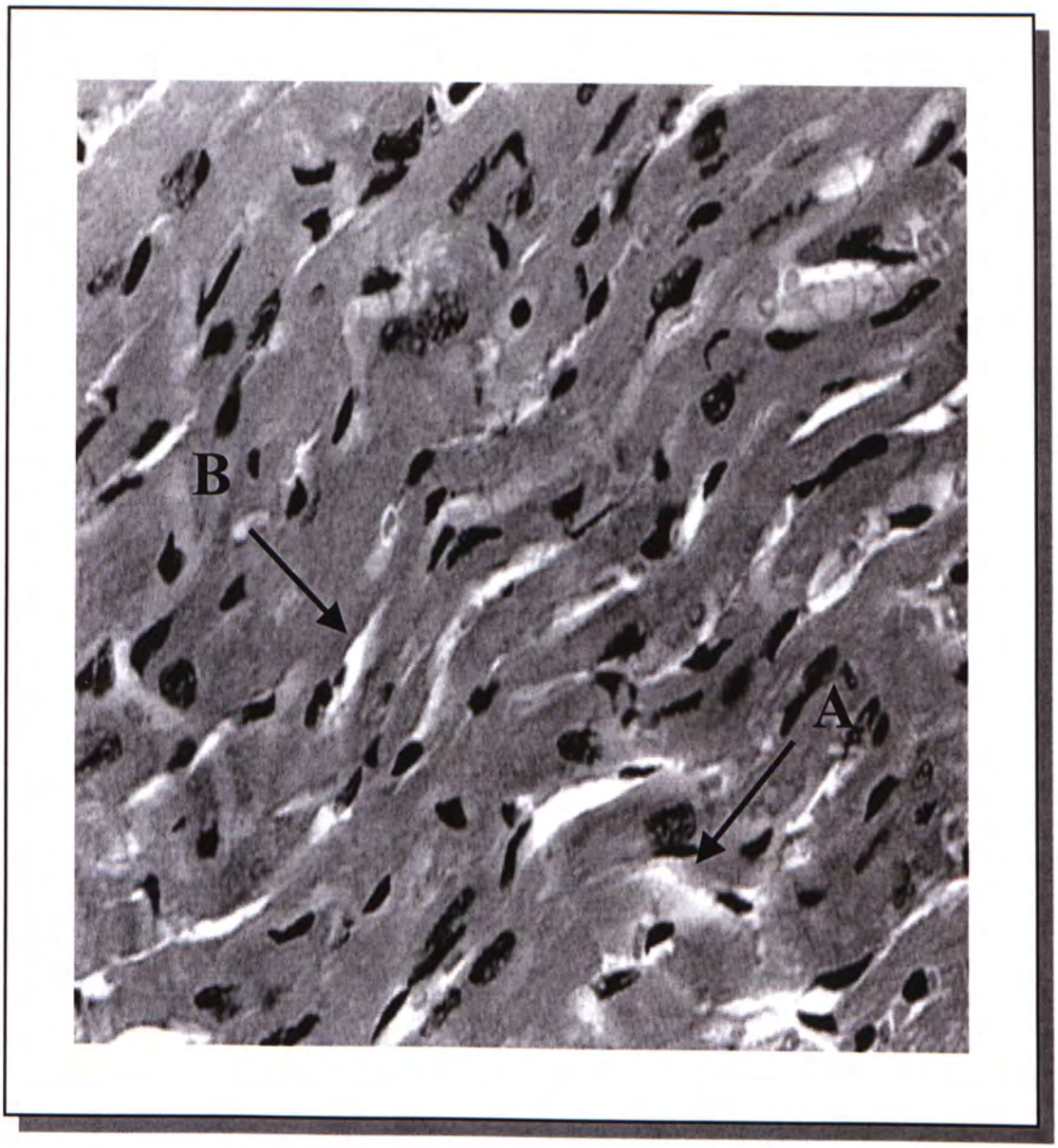


Fig. 3.53. Heart section of R-HepG2 bearing nude mice treated with 2mg/kg Dox
Magnification: 2.5 x 40 x 1 x 4 = 400x
Sample: ventricular wall portion
Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After two days of inoculation, Dox at a dose of 2mg/kg was injected intravenously on every other day. After 4 weeks treatment, the mice were sacrificed by cervical dislocation and the hearts were quickly removed for histological slide preparation. A: the disrupted myocardial filament; B: vancuolization.

3.2.4.5.Heart section of 1mg/kg LDL-Dox treated nude mice bearing R-HepG2 cells

The heart section from the 1mg/kg LDL-Dox treated nude mice bearing R-HepG2 cells was observed under light microscope. LDL-Dox at a dose of 1mg/kg was injected to these mice intravenously on every other day. There was no significant disruption of myocardial filaments and vacuolization found on the heart section when compared with control group (Fig. 3.54).

Fig. 3.54. Heart section of R-HepG2 bearing nude mice treated with 1mg/kg LDL-Dox
Magnification: 2.5 x 40 x 1 x 4 = 400x
Sample: ventricular wall portion
Nude mice were injected subcutaneously with 8 x 10^6 of human resistant hepatoma R-HepG2 cells in the shoulder. After two days of inoculation, LDL-Dox at a dose of 1mg/kg was injected intravenously on every other day. After 4 weeks treatment, the mice were sacrificed by cervical dislocation and the hearts were quickly removed for histological slide preparation.

CHAPTER 4 : DISCUSSION

4.1. *IN VITRO* STUDIES

4.1.1. The cytotoxicity of Dox and LDL-Dox on HepG2 cells and R-HepG2 cells

Results from other workers suggested that the expression level of LDL-receptors on tumor cells was higher than that on normal cells because more LDL would be taken up in tumor cells than that of normal cells for membrane synthesis. Moreover, the patients bearing various cancers are usually found to suffer from hypocholesterolemia and hypolipoproteinemia (Alexopoulos *et al.*, 1987). Regarding to these reasons, LDL is proposed as a target carrier to deliver the anticancer drug to the target tumor cells. This strategy could increase the selectivity of the anticancer drugs to the cancer cells and reduce the adverse side effects to the normal cells.

The results from the quantitative analyses in the accumulation of doxorubicin (Dox) and low density lipoprotein-doxorubicin (LDL-Dox) in HepG2 cells and R-HepG2 cells showed that the LDL-Dox accumulated in the cells was higher than that of free Dox, especially in R-HepG2 cells. These results were consistent with the current literatures studies, i.e. the higher amount of LDL-Dox

accumulated in tumor cells was due to the higher expression level of LDL receptors on their cell membranes.

When comparing the cytotoxic effect of free Dox and LDL-Dox on HepG2 cells and R-HepG2 cells, the results showed that the effect was time-dependent, i.e. the longer drug incubation time, the lower percentage cell survival was observed, as well as dose-dependent, i.e. the higher concentration of drug used, the lower percentage survival was observed. In HepG2 cells, the cytotoxic effect of free Dox was higher than that of LDL-Dox but *vice versa* in R-HepG2 cells. This might imply that in HepG2 cells, Dox might need a longer time to release from the LDL-complex, so the cytotoxic effect of Dox in LDL-Dox was reduced. On the other hand, in R-HepG2 cells, since the size of LDL with diameter of 22nm (Krieger *et al.*, 1979), is bigger than the pore size of P-glycoprotein (P-gp) whose diameter is 5nm, it is difficult for P-gp to bind LDL-Dox and to pump it out. In the case of free Dox, our data indicated that free Dox would be pumped out by P-gp. So the antiproliferative effect of LDL-Dox on R-HepG2 cells was higher than that of free Dox.

4.1.2. The combined treatment on HepG2 cells and R-HepG2 cells

Since prolonged chemotherapy would induce cancer cells developing into resistance to a spectrum of anticancer drugs, the combined treatment would be used to enhance the antiproliferative effects of anticancer drugs in these resistant cancer cells as well as decrease the side effects on normal cells. Two combined treatments, namely hyperthermia to enhance the cell membrane permeability, alter

drug transport activity and the alter cell metabolism (Leunig *et al.*, 1992)) and a Traditional Chinese Medicine, Fructus Craegtus to modulate the LDL receptors, were used to enhance the cytotoxicity of Dox and LDL-Dox on cancer cells.

For the free Dox and hyperthermia treatment, after HepG2 cells and R-HepG2 cells were incubated with free Dox at 43°C, the intracellular level of free Dox was increased. Moreover, the cytotxic effect of free Dox combined with hyperthermia on HepG2 cells was higher than that of free Dox incubated at 37°C. It may be because mild heat would increase the cell membrane permeability and alter the drug transport activity (Leunig *et al.*, 1992). Thus the uptake of free Dox could be enhanced by the cancer cells when combined with hyperthermia and lead to more cytotoxicity on the cancer cells.

When HepG2 cells and R-HepG2 cells were incubated with LDL-Dox at 43°C, the intracellular level of LDL-Dox was similar to that when the cells were incubated with LDL-Dox at 37°C. However, the cytotoxic effect of LDL-Dox combined with hyperthermia was higher than that of LDL-Dox treatment only. The intracellular level of LDL-Dox was not in parallel to the degree of the cytotoxic effect of LDL-Dox when combined with hyperthermia. This might imply that hyperthermia could not enhance the uptake of LDL-Dox, but it might affect the metabolic rate of LDL-Dox in the cells (Leunig *et al.*, 1992), such as increasing the rate of LDL degradation. Therefore, the amount of Dox released from LDL-complex was increased at 43°C when compared with that of the cells at 37°C and lead to as

increase of the cytotoxic effect of LDL-Dox when combined with the hyperthermia treatment.

A Traditional Chinese Medicine, *Fructus Crataegus* (FC), was also used as a combined treatment in present study to increase the antiproliferative effect of LDL-Dox on the tumor cells. Since the components of FC would reduce the serum cholesterol, especially LDL, they may increase the cell expression level of LDL receptors in cells. After the pre-treatment of FC on HepG2 cells, the cytotoxic effect of LDL-Dox was increased when compared with that of the cells treated with LDL-Dox only. Moreover, there was about 15-fold increase of cytotoxic effect of LDL-Dox after FC pre-treatment when compared with that of the LDL-Dox treatment only. This result implied that FC would enhance the LDL receptors on HepG2 cells or increase the binding activity of LDL-Dox to the LDL receptors in order to increase the accumulation of LDL-Dox in the cells and lead to enhance the cytotoxic effect of LDL-Dox. But in R-HepG2 cells, the cytotoxic effect of LDL-Dox could not be increased by the pre-treatment of FC. The mechanism is not known.

4.1.3. The modulation of LDL-R expression

Although the number of LDL receptors on tumor cells were higher than that on normal cells, up-regulation of LDL receptors on tumor cells was also an important strategy when applying LDL-Dox to treat tumor cells. It is because the up-regulation of LDL receptors on tumor cells could increase the specificity of LDL-Dox to the target tumor cells in order to decrease the adverse side effect to the

normal cells. Current studies showed that taurine could enhance the LDL receptor activity (Zouhair *et al.*, 1987), insulin could affect the synthesis of the LDL receptors (Wade *et al.*, 1989). In this project, the Traditional Chinese Medicine, *Fructus Crataegus* (FC), was used to study the possiblity of the up-regulation of LDL receptors on tumor cells.

After the treatment of FC, the expression level of LDL receptors on HepG2 cells was elevated, but not on R-HepG2 cells when compared with that on these cells treated with lipoprotein deficient serum only. Moreover, in HepG2 cells the power of up-regulation of the expression level of LDL receptors by FC was higher than that of LDL-mediated down-regulation of expression level of LDL receptors on tumor cells. It was because when the HepG2 cells were co-treated with LDL and FC, the expression level of LDL receptors was higher than that of the cells treated with LDL only. Thus, the FC could compensate part of the down-regulation of LDL receptors induced by LDL. But the density of LDL receptors on R-HepG2 cells could not be elevated by FC, and it also could not be down-regulated by LDL. The reasons might be that the density of LDL receptors on resistant cells had been saturated, so they were not easily affected by the pre-treatment of FC that was done in order to promote the expression level of LDL receptors. The amount of LDL receptors also could not be down-regulated by LDL because the growth rate of resistant cells was faster than that of the parental cancer cells, so the resistant cells required more LDL for membrane synthesis.

The up-regulation of FC in the LDL receptors expression level on HepG2 cells caused an increase of the accumulation of LDL-Dox in the cells resulting in an increase of the cytotoxic effect of LDL-Dox in these cells. Moreover, the accumulation of LDL-Dox in the cells was decreased when the cells were treated with LDL. It was because most of the LDL receptors might be bound by LDL or the LDL might down-regulate the expression of LDL receptors. The accumulation of LDL-Dox under FC and LDL treatment was lower than that observed under FC pre-treatment only, but higher than that observed under LDL pre-treatment only. The reasons might be that LDL might bind to a part of LDL receptors or diminish a part of LDL receptors expression, so the intracellular level of LDL-Dox was decreased. These results were consistent with the results from the protein expression level of LDL receptors on HepG2 cells after various pre-treatments. In R-HepG2 cells, since the amount of LDL receptors on resistant cells was saturated, so the accumulation of LDL-Dox in the resistant cells was not affected by various treatments. This means that when the resistant cells were incubated with or without FC, LDL or FC and LDL, the intracellular level of LDL-Dox was the same as that when the resistant cells were incubated with lipoprotein deficient serum only. These results were consistent with the results from the protein expression level of LDL receptors on R-HepG2 cells after various pre-treatments with FC. In addition, after the R-HepG2 cells were pre-incubated with FC, the cytotoxic effect of LDL-Dox was not increased when compared with the results when the cells were treated with LDL-Dox only.

4.2 *IN VIVO* STUDIES

It was crucial to demonstrate that LDL-Dox could work as a target carrier to the anticancer drugs and had a better selectivity than free Dox when treating tumor bearing nude mice. To compare the antiproliferative effect of free Dox and LDL-Dox, the tumor size and weight would be measured during different treatments, and to compare the cardiotoxic effect of free Dox and LDL-Dox, the plasma levels of lactate dehydrogenase (LDH) and plasma creatine kinase (CK) would be measured. Moreover, the histological studies would be used as an indicator of the level of heart damage.

Results on parental HepG2 cells bearing nude mice in previous studies showed that the anti-proliferative effect of 1mg/kg LDL-Dox was similar to that of 1mg/kg free Dox on the tumor bearing nude mice, i.e. the tumor weight and tumor size in 1mg/kg LDL-Dox treated group were similar to those in 1mg/kg free Dox treated group. Moreover, in the histogical studies in heart section of 1mg/kg free Dox treated group showed that vacuolization, dilation and disruption of myofibrils arrangement could be observed when compared with the control group which only injected saline. However, the 1mg/kg LDL-Dox treated group did not exhibit any heart damage when compared with the control group. The studies on plasma lactate dehydrogenase (LDH) showed that the Dox-treated group exhibited higher plasma LDH activity when compared with the control group. In contrast, LDL-Dox treated group exhibited similar plasma LDH activity when compared with control group. These results indicated that LDL-Dox would exhibit a similar anti-proliferative effect

on parental HepG2 cells bearing nude mice and reduce the cardiac toxicity induced by free Dox (Chu *et al.*, in press).

The studies on the tumor size and weight in R-HepG2 bearing nude mice in present studies showed that the tumor size and weight in 1mg/kg Dox-treated group was similar to that in the control group. But the tumor size and weight in 2mg/kg Dox-treated group was lower than that in control group. These results showed that in R-HepG2 cells bearing nude mice, the lower concentration of Dox did not exhibit any antitumor effect because the existence of P-glycoprotein in the resistant cells would decrease the concentration of free Dox in the target cells. Moreover, when higher dose of Dox was used, although the P-glycoprotein would pump out free Dox, by proportion, the concentration of free Dox in the target cells was higher than that when lower dose of Dox was used. Therefore, the antiproliferative effect of 2mg/kg Dox treatment was higher than that of 1mg/kg Dox treatment. These results were consistent with those from the percentage survival of R-HepG2 cells on Dox treatment because the IC_{50} of free Dox for 48 hours treatment was 300μM. In addition, the tumor size and weight in 1mg/kg LDL-Dox treated group was lower than that in control group and 1mg/kg Dox-treated group. These results implied that the antiproliferative effect of 1mg/kg LDL-Dox treatment was higher than that of 1mg/kg Dox treatment. Moreover, the size and weight of tumor in 1mg/kg LDL-Dox treated group was similar to that in 2mg/kg Dox treated group. This result showed that the antitumor effect of LDL-Dox was higher than that of free Dox because after the Dox was coupled into the LDL, the dose of Dox used in LDL-Dox was lower than that of free Dox used. This implied that after Dox was coupled

into LDL, the LDL-Dox so formed was not easily to be pumped out by the P-glycoprotein because of the size of LDL was larger than the pore size of P-glycoprotein. This result was consistent to that from the percentage of cells survival on free Dox and LDL-Dox treatments, i.e. the cytotoxic effect of LDL-Dox, in which IC_{50} was 15μM for 48 hours, was higher than that of free Dox, in which IC_{50} was 300μM for 48 hours, on the resistant cells. These results also demonstrated that the targeted effect of LDL-complex to the tumor cells because the lower dosage of LDL-Dox could exhibit the same antitumor effect on the tumor-bearing mice treated at higher dosage of free Dox.

Reports from the literatures showed that the major side effects of Dox were cardiomyopathy and congestive heart failure (Singal *et al.*, 1997). The results from histological studies of heart section showed that in the nude mice which did not receive subcutaneously of R-HepG2 cells (none group), the mycocardial filament were well organized and tightly packed and this phenomenon was also observed in the control tumor-bearing mice group which were only injected with normal saline during the 4 weeks treatment. This result implied that saline would not affect the organization of mycocardial filament. But in the Dox treated groups, including treatment of 1mg/kg and 2mg/kg free Dox, the vacuolization, dilation and disruption of myofibrils arrangement in the heart sections could be observed in the R-HepG2 bearing nude mice when compared to the control group. This result shows that free Dox could induce cardiac toxicity. In contrast, LDL-Dox treated mice showed that there was no clear vacuolization, dilation and disruption of myofibrils arrangement in the heart section when compared to that of the control group. Thus this result implies

that LDL-Dox exerted less cardiac toxicity than free Dox, and 1mg/kg LDL-Dox treatment exhibited similar antiproliferative effect of 2mg/kg free Dox treatment.

The studies on plasma lactate dehydrogenase (LDH) activity and creatine kinase (CK) activity in nude mice would also demonstrate the level of heart damage induced by different kind of treatments. It was because when heart damage took place, the level of plasma LDH and CK would significantly increase. The plasma LDH activity in none group was similar to that in the saline group, suggesting that saline did not induce the cardiac toxicity effect. After the tumor bearing mice were treated with free Dox, at high and low dose of Dox, the plasma LDH activity in both treated groups were higher when compared with that of the control group. Moreover, the plasma LDH activity at high dose treatment was higher than that at low dose treatment, i.e. the higher concentration of free Dox used, the higher degree of cardiac toxicity would result. In contrast, the tumor bearing mice treated with 1mg/kg LDL-Dox showed a plasma LDH activity similar to that of the control group. This result suggested that the LDL-Dox-induced heart damage was much lower than that induced by free Dox. These results were consistent to those from the histological studies of heart section, i.e. free Dox could induce cardiac toxicity but LDL-Dox treatment could not induce any cardic toxicity.

For the measurement of plasma CK activity in different groups, the none group and control group showed a similar plasma CK activity. In addition, in LDL-Dox treated tumor bearing mice, the plasma CK activity was similar to that of the control group. Thus, this result indicated that the LDL-Dox exerted less cardiac

toxicity. These results were consistent to the results from the plasma LDH activity measurement. In contrast, the levels of plasma CK activity in Dox-treated group, treated with 1mg/kg free Dox or 2mg/kg free Dox, were both higher when compared with those of the control group. Also the plasma CK activity in group with high dose free Dox treatment was higher than that in low dose free Dox treatment. This result means the heart damage was induced by free Dox treatment and the level of heart damage was dose-dependent, i.e. the higher concentration of Dox used, the higher level of cardiac toxic effect. This result was also consistent to the result from the plasma LDH activity measurement.

The aforesaid results gave more solid evidence on the reduction of the cardiac toxic side effect by using LDL as Dox carrier. In addition, it showed that lower dose of LDL-Dox could have the similar antiproliferative effect as high dose free Dox treatment in R-HepG2 bearing mice *in vivo*, that means LDL-Dox could circumvent multidrug resistance in these resistant cells.

CHAPTER 5 : CONCLUSION

5.1. CONCLUSION

5.1.1. *In vitro* studies

In the present studies, our results have demonstrated that low density lipoprotein (LDL) can work as a drug carrier to the target tumor cells. The antitumor drug, doxorubicin (Dox) was coupled with the LDL without altering the structure of LDL. Moreover, the intracellular level of LDL-Dox in HepG2 cells and R-HepG2 cells was found to be higher than that of free Dox. Although the cytotoxic effect of LDL-Dox was lower in the HepG2 cells when compared with the free Dox at the same dose, its cytotoxicity was higher in R-HepG2 cells when compared with the free Dox *in vitro*. It shows that LDL-Dox is able to circumvent the resistant cells because the size of LDL is bigger than the pore of P-glycoprotein, so it is difficult for P-glycoprotein to pump the LDL out of the cells.

In addition, modulation of LDL receptors on tumor cells is also important because it can increase the population of LDL receptors on tumor cells in order to increase the accumulation of LDL-Dox in the cells. The studies found that a Traditional Chinese Medicine, *Fructus Crataegus* (FC), can elevate the expression level of LDL receptors on HepG2 cells in order to increase the intracellular level of LDL-Dox resulting in increase of the cytotoxic effect of LDL-Dox on HepG2 cells

when compared with that of the LDL-Dox treatment only. Moreover, the power of FC in elevating the LDL receptors on HepG2 cells is higher than that of LDL down-regulating the LDL receptors on tumor cells. In contrast, the Traditional Chinese Medicine, FC, and the existence of LDL did not affect the density of LDL receptors on R-HepG2 cells and the intracellular level of LDL-Dox in the cells. So these results suggest that the amount of LDL receptors on the resistant cells were not affected by these two factors. This difference is because the growth rate of R-HepG2 cells is faster than that of parental HepG2 cells, so the amount of LDL used for membrane synthesis is more than that of the parental cells and the LDL receptors on resistant cells is higher. The conclusions of the current studies are that FC induces the LDL receptors activity on parental HepG2 cells but not on R-HepG2 cells. Therefore, parental cells can accumulate more LDL-Dox and lead to higher cytotoxicity.

Hyperthermia is another strategy to increase the efficiency of free Dox and LDL-Dox. In HepG2 cells and R-HepG2 cells, as the free Dox combined with hyperthermia, the intracellular level of free Dox is increased. It is because hyperthermia is able to increase the cell membrane permeability and then lead to more cytotoxicity. Although in HepG2 cells and R-HepG2 cells, the accumulation of LDL-Dox cannot be enhanced by hyperthermia, the cytotoxic effect of LDL-Dox is increased when combined with hyperthermia. It is because heat may alter the cell metabolic rate in order to enhance the degradation of LDL and then release the free Dox.

5.1.2. *In vivo* studies

It is important to demonstrate that LDL can serve as a drug carrier to the target tumor cells in the clinical trail. The nude mice bearing R-HepG2 cells were treated with saline, 1mg/kg Dox , 2mg/kg Dox and 1mg/kg LDL-Dox. After four weeks treatment, the tumor size and weight in descending order is as follows: saline, 1mg/kg Dox, 2mg/kg Dox, 1mg/kg LDL-Dox. This result indicate that lower concentration of free Dox does not have any significant antiproliferative effect in the R-HepG2 bearing nude mice. But in the higher dose of free Dox, its antiproliferative effect is similar to that of 1mg/kg LDL-Dox treatment. It implies that the antitumor effect of LDL-Dox is higher than that of the free Dox, i.e. the low dose of LDL-Dox used has the same antiproliferative effect as that of a higher dose of free Dox used.

By observing the histological section of hearts in R-HepG2 bearing nude mice, the pattern of myocardial filament in none group and control group is the same which indicated that saline does not cause the damage of heart. Moreover, only the Dox treatment, no matter at 1mg/kg free Dox or 2mg/kg free Dox, caused the disruption and vacuolization of myocardial filament. But this damage is not found in mice treated with LDL-Dox. In addition, the plasma LDH activity and plasma CK activity was higher in the Dox-treated mice when compared with those of LDL-Dox treated mice and the control group. These results further prove that LDL-Dox does not exert severe cardiac toxicity like the free Dox. Furthermore, the antiproliferative effect of LDL-Dox is similar to that of the higher dose of free Dox treatment on R-HepG2 bearing nude mice.

5.2. FUTURE PROSPECTIVE

Although LDL-Dox has better selectivity towards tumor cells and decreases the side effect, it is still important to improve the antitumor effect of LDL-Dox. According to the *in vitro* result, it shows that the Traditional Chinese Medicine, *Fructus Crataegus* (FC), can elevate the LDL receptor expression, so this effect would need to be studied concerning the effects of FC on the regulation of the LDL receptors activity in HepG2 bearing nude mice. Moreover, it is important to prove that FC only can induce the expression level of LDL receptors in tumor cells, but not in normal tissues.

In addition, the combined treatment of hyperthermia and LDL-Dox could increase the release of free Dox from LDL-complex in the tumor (HepG2 cells and R-HepG2 cells) bearing nude mice in order to increase the antiproliferative effect of LDL-Dox. Since the combined treatment of hyperthermia and free Dox could increase the uptake by the tumor cells, this strategy need to be proven in the tumor bearing nude mice.

Since the model in the current studies is to use nude mice with the injection of tumor cells subcutaneously, it is important to create a model in which the liver cancer cells are located in the liver, which is more clinically relevant to investigation of the LDL-Dox cytotoxicity on liver cancer. In that model the tumor cells may be implanted into the spleen of nude mice in which the cells can be transported to the liver through hepatic portal circulation and the drug should be injected intravenously.

Last but not least, there is a limitation in the concentration of the LDL-Dox preparation. The method used in the current studies is about 300μM at maximum. This maximum concentration limits the dose of treatment to tumor bearing mice at a lower concentration. Therefore we need to enhance the coupling efficiency in order to use a higher dose of drug for the investigation to examine whether higher dose of LDL-Dox may exert any adverse side effects.

REFERENCES

Akuta K., Abe M., Kondo M., Yoshikawa T., Tanaka Y., Yoshida M., Miura T., Nakao N., Onoyama Y., Yamada T., Mukoujima T., and Tsukada K. (1991). Combined effects of hepatic arterial embolization using degradable starch microspheres (DMS) in hyperthermia for liver cancer. International Journal of Hyperthermia 7: 231 – 242.

Alexopoulos C. G., Blatsios B., and Avgerinos A. (1987). Serum lipids and lipoprotein disorders in cancer patients. Cancer 60: 3065 – 3070.

Angelin B., Raviola C. A., Innerarity T. L., and Mahley R. W. (1983). Rapid regulation of apolipoprotein B, E receptors, but not of apolipoprotein E receptors, by intestinal lipoproteins and bile acids. The Journal of Clinical Investigation 71: 816.

Armour E. P., McEachern D., Wang Z., Corry P. M., Martinez A. (1993). Sensitivity of human cells to mild hyperthermia. Cancer Research 53: 2740-2744.

Baboli M. S., Favre G., Blancy E., and Soula G. (1989). Preparation of low density lipoprotein-9-methoxy ellipticin complex and its cytotoxcity effect against L1210 and P388 leukemic cells *in vitro*. European Journal of Cancer and Clinical Oncology 25, 233 – 241.

Baboli M. S., Favre G., Canal P., and Soula G. (1993). Low density lipoprotein for cytotoxic drug targeting: improved activity of elliptinium derivative against B16 melanoma in mice. The British Journal of Cancer 68: 319 – 326.

Bachur N. R., Gordon S. L., and Gee M. W. (1977). Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. Molecular Pharmacology 13: 901 – 910.

Bachur N. R., Yu F., and Johnson R. (1992). Helicase inhibition by anthracycline anticancer agents. Molecular Pharmacology 41: 993 – 998.

Barrand M. A., Rhodes T., Center M. S., and Twentyman P. R. (1993). Chemosensitization and drug accumulation effects of cyclosporin A, PSC-833, and verapamil in human MDR large cell lung cancer cells expressing a 190k membrance protein distinct from P-glycoprotein. European Journal of Cancer 29: 408 – 415.

Bilheimer D.W. (1988). The lipoprotein receptor concept. Drug 36: 55 – 62.

Brown M. S., Dana S. E., and Goldetein J. L. (1975). Cholesterol ester formation in culture human fibroblasts. The Journal of Biological Chemistry 250: 4025 – 4027.

Budd D., and Ginsberg H. (1986). Hypocholesterolemia and acute myelogenous leukemia . Assoication between disease activity and plasma low-density lipoprotein cholesterol concentrations. Cancer 58: 1361 – 1365.

Calderwood S. K., Hahn G. M. (1983). Thermal sensitivity and resistance of insulin-receptor binding. International Journal of Biochemistry and Biophysics 756: 1 - 8.

Cambien F., Duciemetiere P., and Richard J. (1980). Total serum cholesterol and cancer mortality in a middle-aged male population. American Journal of Epidemiology 112: 388 – 394.

Cardo C. C., O'Brien J. P., and Casals D. (1989). Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. National Academy Sciences (U.S.) 86:695-8.

Chappell D. A., Fry G. L., Waknitz M. A., Muhonen L. E., and Pladet M. W. (1993). Low density lipoprotein receptors bind and mediate cellular catabolism of normal very low density lipoprotein in vitro. The Journal of Bioliogy Chemistry 268: 25487 – 25493.

Chu C. Y. A., Tsang S. Y., Lo H. K. E., and Fung K. P. Low density lipoprotein as a targeted carrier for doxorubicin in nude mice bearing human hepatoma HepG2 cells. Life Science (in press).

Chu Y. F. (1988). The extraction of fructus crataegus would affect the cholesterol level in plasma. The Traditional Chinese Medicine. 19: 25 – 27.

Danø K. (1973). Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. International Journal of Biochemistry and Biophysics 323: 466 - 483.

Dashti N., Wolfbauer G., Koren E., Knowles B. and Alaupovic P. (1984). Catabolism of human low density lipoproteins by human hepatoma cell line HepG2. Biochimica et Biophysica Acta 794: 373 – 384.

De Smidt P.C. and Van Berkel T. J. C. (1990). Prolong serum half-life of antineoplastic drugs by incorporation into the low density lipoprotein. Cancer Research 50: 7476 – 7482.

Doroshow J. H., Akman S., Esworthy S., Chu F. F., and Burke T. (1991). Doxorubicin resistance conferred by selective enhancement of intracellular glutathione peroxidase or superoxide dismutase content in human MCF-7 breast cancer cells. Free Radical Research 12: 779 – 781.

Doroshow J. H., Locker G. Y., and Myers C. E. (1980). Enzymatic defenses of the mouse heart against reactive oxygen metabolites: alterations produced by doxorubicin. The Journal of Clinical Investigation 65:128 - 135.

Dubowchik G. M. and Firestone R. A. (1995). Improved cytotoxicity of antitumor compounds deliverable by the LDL pathway. Bioconjugate Chemistry 6: 427 – 439.

Fardel O., Lecureur V., and Guillouzo A. (1996). The P-glycoprotein multidrug transporter. General Pharmacology 27: 1283 – 1291.

Feinleib M. (1983) Review of the epidemiological evidence for a possible relationship between hypocholesterolemia and cancer. Cancer Research 43: 2503 – 2507.

Filipowska D., Filipowski T., Morelowska B., Kazanowska W., Laudanski T., Lapinjoki S., Akerlund M., and Breeze A. (1992). Treatment of cancer patients with a low-density-lipoprotein delivery vehicle containing a cytotoxic drug. Cancer Chemotherapy and Pharmacology 29: 396 – 400.

Flora S. D., D'Agostini F., Masiello L., Giunciuglio D., and Albini A. (1996). Synergism between N-acetylcysteine and doxorubicin in the prevention of tumorigenicity and metastasis in murine model. International Journal of Cancer 67: 842 – 848.

Fogleson P. D., Reckord C., and Swink S. (1992). Doxorubicin inhibits human DNA topoisomerase I. Cancer Chemotherapy and Pharmacology 30: 123 – 125.

Gao M., Yamazaki M., Loe D. W., Westlake C. J., Grant C. E., Cole S. P. C., and Deeley R. G. (1998). Multidrug resistance protein. The Journal of Biological Chemistry 273: 10733 – 10740.

Gerke A., Westesen K., and Koch H. J. (1996). Physicochemical characterization of protein-free low density lipoprotein models and influence of drug loading. Pharmaceutical Research 13: 44 – 51.

Giavazzi R., Jessup J. M., Campbell D. E., Walker S. M. and Fidler I. J. (1986). Experimental nude mouse model of human colorectal cancer liver metastases. Journal of the National Cancer Institute 77: 1303 – 1407.

Gnant M. F. X., Noll L. A., Terril R. E., Wu P. C., Berger A. C., and Nguyen H. Q. (1999). Isolated hepatic perfusion for lapine liver metastases: impact of hyperthermia on permeability of tumor neovasculature. Surgery 126: 890 - 899.

Gottesman M. M. and Pastan I. (1988). The multidrug-transporter: a double-edged sword. The Journal of Biology Chemistry 263: 12163 – 12166.

Hagasawa K., Takara K., Nomiyama M., Ohnishi N., and Yokoyama T. (1996). Transport mechanisms of anthracycline derivatives in human leukemia cell lines: uptake of pirarubicin, daunorubicin and doxorubicin by K562 and multidrug-resistant K562/ADM cells. Biological and Pharmaceutical Bulletin 19: 971 – 976.

Halbert G. W., Stuart J. F. B., and Florence A. T. (1985). A low density lipoprotein-methotrexate covalent complex and its activity against L1210 cells in vitro. Cancer Chemotherapy and Pharmacology 15: 223 – 227.

Henriksson P., Eriksson M., Ericsson S., Rudling M., Stege R., Berglund L., and Angelin B. (1989). Hypocholesterolaemia and increased elimination of low-density lipoproteins in metastatic cacner of the prostate. Lancet ii: 1978 – 1180.

Ho Y. K., Smith R. G., Brown M. S., and Goldstein J. L. (1978). Low density lipoprotein (LDL) receptor activity in human acute myelogenous leukemia cells. Blood 52: 1099 – 1114.

Horio M., Gottesman M. M., and Pastan I. (1988). ATP-dependent transport of vinblastine in vesicles from human multidrug-resistance cells. National Academy of Sciences (U.S.A.) 85: 3580 – 3584.

Hynds S. A., Welsh J., Stewart J. M., Jack A., Soukop M., McArdle C. S., Calman K. C., Packard C. J., and Shepherd J. (1984). Low-density lipoprotein metabolism in mice with soft tissue tumors. International Journal of Biochemistry and Biophysics 795: 589 - 595.

Hynds S., Welsh J., Stewart J. M., Jack A., Soukop M., Mcardle C. S., Calman K. C., Packard C. J., and Shepherd J. (1984). Low-density lipoprotein metabolism mice with soft tissue tumors. Biochimica et Biophysica Acta 795: 589 – 595.

Iwanik M. J., Shaw K. V., Ledwith B. J., Yanovich S., and Shaw J. M. (1984). Preparation of interaction of a low-density lipoprotein: daunomycin complex with P388 leukemic cells. Cancer Research 44: 1206 – 1215.

Izem L., Rassart E., Kamate L., Falstrault L., Rhainds D., and Brissette L. (1998). Effect of reduced low-density lipoprotein receptor level on HepG2 cell cholesterol metabolism. The Biochemical Journal 329: 81 – 89.

Johnson R. K. and Goldin A. (1975). The clinical impact of screening and other experimental tumor studies. Cancer Treatment Reviews 2: 1 - 7.

Jones P. M. and George A. M. (1998). A new structural model for P-glycoprotein. The Journal of Membrane Biology 166: 133 – 147.

Jones P. M. and George A. M. (2000). Symmetry and structure in P-glycoprotein and ABC transporters. What goes around come around. European Journal of Biochemistry 267: 5298 – 5305.

Kim R., Hirabayashi N., Nishiyama M., Saeki S., Toge T., and Okada K. (1991). Expression of MDR1, GST-π and topoisomerase II as an indicator of clinical response to adriamycin. Anticacner Research 11: 429 – 432.

Klock J. C., and Pieprzyk J. K. (1979). Cholesterol, phospholipids, and fatty acids of normal immature neutrophils: comparison with acute myeloblastic leukemia cells and normal neutrophils. Journal of Lipid Research 20: 908 – 911.

Koseki Y, Israel M., and Sweatman T. W. (1991). Inhibitory effects of immobilized adriamycin on cell growth and thymidine uptake in human CEM leukemic lymphocytes. Journal of Cell Pharmacology 2: 171 – 178.

Kramer R. A., Zakher J., and Kim G. (1988). Role of the glutathione redox cycle in acquired and de novo multidrug resistance. Science 24: 694 – 697.

Krieger M., Brown M. S., Faust J. R., and Goldstein J. L. (1978). Replacement of endogenous cholestery esters of low density lipoprotein with exogenous cholesteryl linoleate. The Journal of Biological Chemisty 253: 4093 – 4101.

Krieger M., Smith L. C., Anderson R. G. W., Goldstein J. L., Kao Y. J., Pownall H. J., Gotto A. M. J., and BrownM. S. (1979). Reconstituted low density lipoprotein: a vehicle for the delivery of hydrophobic fluorescent probe to cells. Journal of Supramolecular Structure 10: 467 – 478.

Krieger M., McPhaul M. J., Goldstein J. L., and Brown M. S. (1979). Replacement of netural lipids of low density lipoprotein with esters of long chain unsaturated fatty acids. The Journal of Biological Chemistry 254: 3845 - 3853.

Lafreniere R. and Rosenberg S. A. (1986). A novel approach to the generation and identification of experimental hepatic metastases in a murine model. Journal of the National Cancer Institute 76: 309 – 322.

Lefrak E. A., Pitha J., Rosenheim S., and Gottlieb J. A. (1973). A clinicopathologic analysis of Adriamycin cardiotoxicity. Cancer 32: 302 - 314.

Lehninger A. L., Nelson D. L., and Cox M. M. (1993). Principles of Biochemistry 2nd Edition. Worth Publishers: 675 – 678.

Lepock J. R. (1982). Involvement of membranes in cellular responses to hyperthermia. Radiation Research 92: 433 - 438.

Leunig M., Goetz A. E., Dellian M., Zetterer G., Gamarra F., and Jain R. K. (1992). Interstitial fluid pressure in solid tumors following hyperthermia: possible correlation with therapeutic response. Cancer Research 52: 487 - 490.

Lew Y. S., Brown S. L., Griffin R. J., Song C. W., and Kim J. H. (1999). Arsenic trioxide causes selective necrosis in solid murine tumors by vascular shutdown. Cancer Research 59: 6033 – 6037.

Ling Y. H., Priebe W., and Soler R. P. (1993). Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug rsisitance cells. Cancer Research 53: 1583 – 1589.

Lundberg B. (1987). Preparation of drug-low density lipoprotein complexes for delivery of antitumoral drugs via the low density lipoprotein pathway. Cancer Research 47: 4105 – 4108.

Lundberg B. (1992). Assembly of prednimustine low-density-lipoprotein complexes and their cytotoxic activity in tissue culture. Cancer Chemotherapy and Pharmacology 29: 241 – 247.

Mankertz J., Nundel M., Baeyer H. V., and Riedel E. (1997). Low density lipoprotein as drug carriers in the therapy of macrophage-associated diseases. Biochemical and Biophysical Research Communications 240: 112 – 115.

Masquelier M., Vitols S., and Peterson C. (1986). Low-density lipoprotein as a carrier of antitumoral drugs: in vivo fate of drug-human low-density lipoprotein compleses in mice. Cancer Research 46: 3842 - 3847.

Morrow C. S., and Cowan K. H. (1990). Glutathion S-transferase and drug resistance. Cancer Cells 2: 15 – 22.

Muller H. G. (1939). Cholesterol metabolism in health and anemia. Medicine 9: 119 – 174.

Murphree S. A., Cunningham L. S., Hwang K. M., and Sartirelli A. C. (1976). Effects of adriamycin on surface properties of sarcoma 180 ascites cells. Biochemical Pharmacology 25: 1227 – 1231.

Nagels H., Gebhardt A., Niendorf A., Kroschinski J., and Zeller W. (1997). LDL receptor activity in human leukocyte subtypes: regulation by insulin. Clinical Biochemistry 30: 531 – 538.

Nakagawa T., Ueyama Y., Nozaki S., Yamashita S., Menju M., Funahashi T., Takemura K. K., Kubo M., Tokunaga K., Tanaka T., Yagi M., and Matsuzawa Y. (1994). Marked Hypocholesterolemia in a Case with Adrenal Adenoma – Enhanced Catabolism of Low Density Lipoprotein (LDL) via the LDL Receptors of Tumor Cells. Journal of Clinical Endocrinology and Metabolism 80: 92 – 96.

Oleson J. R. (1995). Hyperthermia from the clinic to the laboratory: a hypothesis. International Journal of Hyperthermia 11: 315 – 322.

Overgaard J. (1989). The current and potential role of hyperthermia in radiotherapy. International Journal of Radiation Oncology Biology Physics 16: 535 - 549.

Parini P., Angelin B. Q., Lobie P. E., Norstedt G., and Rudling M. (1995). Growth hormone specifically stimulates the expression of low density lipoprotein receptors in human hepatoma cells. Endocrine Research 136: 3767 – 3773.

Pereira E., Tarasiuk J., and Suillerot A. G. (1998). Kinetic analysis in living cells of inhibition of P-glycoprotein-mediated efflux of anthracyclines by vinca alkaloids. Chemico-Biological Interactions 114: 61 – 76.

Piero A. D., Dayan G., Consell G., Steinfels E., Krell T., Trompier D., Cortay H. B., and Jault J. M. (1999). P-glycoprotein-mediated resistance to chemotherapy in cancer cells: using recombinant cytosolic domains to establish structure-function relationships. Brazilian Journal of Medical and Biological Research 32: 925 – 939.

Rayyes O. A. and Floren C. H. (1998). Troglitazone upregulates LDL receptor activity in HepG2 cells. Diabetes 47: 1193 – 1198.

Reddy B. S. (1981). Dietary fat and its relationship to a large bowel cancer. Cancer Research 41: 3700 – 3705.

Roninson I. B., Chin J. E., and Choi K. (1986). Isolation of human mdr DNA sequences amplified in multidrug-resistance KB carcinoma cells. National Academy of Sciences (U.S.A.) 83: 4538 – 4542.

Rope P. D. (1997). The role of the MDR protein in altered drug translocation across tumor cells membranes. International Journal of Biochemistry and Biophysics 1241: 385 – 405.

Rose G. and Shipley M. J. (1980). Plasma lipids and mortality: A source of error. Lancet I: 523 – 526.

Rosen G. M., and Halpern H. J. (1990). Spin trapping biologically generated free radicals: correlating formation with cellular injury. Methods in Enzymology 18: 611-621.

Rudling M. J., Collins V. P., and Peterson C. O. (1983). Delivery of aclacinomycin A to human glioma cells in vitro by the low-density lipoprotein pathway. Cancer Research 43: 4600 – 4605.

Rumsey S. C., Galeano N. F., Arad Y., and Deckelbaum R. J. (1992). Cryopreservation with sucrose maintains normal physical and biological properties of human plasma low density lipoproteins. Journal of Lipid Research 33: 1551 – 1561.

Rumsey S. C., Stucchi A. F., Nicolosi R. J., Ginsbery H. N., Ramakrishnan R., and Deckelbaum R. J. (1994). Human plasma LDL cryopreserved with sucrose maintains in vivo kinetics indistinguishable from freshly isolated human LDL in cynomolgus monkeys. Journal of Lipid Research 35: 1592 – 1598.

Saxena K. and Shipley G. G. (1997). Structural studies of detergent-solubilized and vesicle-reconstituted low-density lipoprotein (LDL) receptor. Biochemistry 36: 15940 – 15948.

Shakil A., Osborn J. L., and Song C. W. (1999). Changes in oxygenation status and blood flow in a rat tumor model by mild temperature hyperthermia. International Journal of Radiation Oncology Biology Physics 43: 859-65.

Shimano H., Aburatani H., Mori N., Ishibashi S., Gotohda T., Mokuno H., Kawakami M., Akanuma Y., Takaku F., Murase T., and Yamada N. (1988). Dowregulation of hepatic LDL receptor protein and messenger RNA in fasted rabbits. The Journal of Biochemistry 104: 712 – 718.

Singal P. K., Deally C. M. R., and Weinberg L. E. (1987). Subcellular effects of Adriamycin in the heart: a concise review. Journal of Molecular and Cellular Cardiology19: 817 - 828.

Singal P. K., Iliskovic N. S., Hill M., Thomas T. P., Li T. (1995). Combination therapy with probucol prevents Adriamycin-induced cardiomyopathy. Journal of Molecular and Cellular Cardiology 27: 1055 - 1063.

Singal P. K., Iliskovic N., Li T., and Kumar D. (1997). Adriamycin cardiomyopathy: pathophysiology and prevention. The FASEB Journal 11: 931- 936.

Sinha B. K., Katki A. G., Batist G., Cowan K. H., and Myers C. E. (1987). Adriamycin-stimulated hydroxyl radical formation in human breast tumor cells. Biochemical Pharmacology 36:793-796.

Sit M. W., Tai L. H., and Hsieh Y. Y. (1985). The Chromatograghy and determination of crataegus. The Traditional Chinese Medicine. 16: 37 – 45.

Slapak C. A., Martell R. L., Terashima M., and Levy S. B. (1996). Increased efflux of vincristine, but not of daunorubicin, associated with the murine multidrug resistance protein (MRP). Biochemical Pharmacology 52: 1569 – 1576.

Smit J. W., Duin E., Steen H., Oosting R., Roggeveld J., and Meijer D. K. F. (1998). Interactions between P-glycoprotein substrates and other cationic drugs at the hepatic excretory level. British Journal of Pharmacology 123: 361 – 370.

Smyth M. J., Pietersz G. A., and McKenzie I. F. C. (1987). Specific targeting of chlorambucil to tumor with the use of monoclonal antibodies. Cancer Research 47: 62 – 67.

Stratmann G., Jarosch A., Sulzbacher A., Schuler R., Kumel G., and Woodcock B. G. (1997). Transmembranous transport of drugs: implications for kinetics and drug action. International Journal of Clinical Pharmacology and Therapeutics 35: 151 – 154.

Tatidis L., Gruber A., and Vitols S. (1997). Decreased feedback regulation of low density lipoprotein receptor activity by sterols in leukemic cells from patients with acute myelogenous leukemia. Journal of Lipid Research 38: 2436 – 2445.

Tewey K. M., Rowe T. C., and Yang L. (1984). Adriamycin-induced DNA damage mediated by mammalian DNA topisomerase II. Science 226: 466 – 468.
Thiebaut F., Tsuruo T., Hamada H., Gottesman M. M., Pastan I., and Willingham M. (1987). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. National Academy of Sciences (U.S.A.) 84:7735 - 7738.

Tokui T., Kuroiwa C., Muramatsu S., Tokui Y., SasagawaK., Ikeda T., and Komai T. (1995). Plasma lipoproteins as targeting carriers to tumour tissues after

administration of a lipophilic agent to mice. Biopharmaceutics and Drug Disposition 16: 91 – 103.

Tokui T., Takatori T., Shinozaki N., Ishigami M., Shiraishi A., Ikeda T. and Tsuruo T. (1995). Delivery and cytotoxicity of RS-1541 in St-4 human gastric cancer cells in vitro by the low-density-lipoprotein pathway. Cancer Chemotherapy and Pharmacology 36: 1 – 6.

Tokui T., Tokui Y., Ishigarmi M., Tanzawa K., Ikeda T., and Komai T. (1994). Targeting of antitumor agent, RS-1541 (palmitoyl-rhizoxin), via low-density lipoprotein receptor. International Journal of Pharmaceutics 110: 277 – 283.

Tritton T. R. (1991). Cell surface actions of Adriamycin. Pharmacology and Therapeutics 49: 293 – 309.

Upeslacis J., and Hinman L. (1988). Chemical modification of antibodies for cancer chemotherapy. Annual Reports in Medicinal Chemistry 23: 151 – 160.

Venkatanarayanan S. and Nagarajan B. (1988). Association between tumor status and serum lipoprotein cholesterol in hemopoietic malignancy. Biochemistry International 17: 499 – 507.

Versluis J. A., Rump E. T., Rensen T. C. N., BerkelJ. C. V., and Bijsterbosch M. K. (1999). Stable incorporation of a lipophilic daunorubicin prodrug into apolipoprotein E-exposing liposomes induces uptake of prodrug via low-density lipopotein receptor in vivo. The Journal of Pharmacology and Expermental Therapeutics 289: 1 – 7.

Vitols S. (1991). Uptake of low density lipoprotein by malignant cells: possible therapeutic applications. Cancer Cells 3: 488-95.

Vitols S., Angelin B., Ericsson S., Gahrton G., Juliusson G., Masquelier M., Paul C, Peterson C., Rudling M., Reid K. S., and Tidefelt U. (1990). Uptake of low density lipoproteins by human leukemic cells in vivo: Relation to plasma lipoprotein levels and possible relevance for selective chemotherapy. National Academy of Sciences (U.S.A.) 87: 2598 – 2602.

Vitols S., Gahrton G. and Peterson C. (1984). Significance of the low density lipoprotein (LDL) receptor pathway for the *in vitro* accumulation of AD-32 incorporated into in normal and leukemic white blood cells. Cancer Treatment Reports 68: 515 – 520.

Vitols S., Reid K. S., Masquelier M., Sjostrom B. and Peterson C. (1990). Low density lipoprotein for delivery of a water-insoluble alkylating agent to malignant cells. *In vitro* and *in vivo* studies of a drug-lipoprotein complex. The British Journal of Cancer 62: 724 – 729.

Wade D. P., Knight B. L., and Soutar A. K. (1988). Hormonal regulation of low-density lipoprotein (LDL) receptor activity human hepatoma HepG2 cells. Insulin

increases LDL receptor activity and diminishes its suppression by exogenous LDL. European Journal of Biochemistry 174: 213 – 218.

Wade D. P., Knight B. L., and Soutar A. K. (1989). Regulation of low-density lipoprotein-receptor mRNA by insulin in human hepatoma HepG2 cells. European Journal of Biochemistry 181: 727 – 731.

Wadkins R. M., and Roepe P. D. (1997). Biophysical aspects of P-glycoprotein-mediated multidrug resistance. International Review Cytol. 171: 121 – 165.

Westesen K., Gerke A., and Koch M. H. J. (1995). Characterization of native and drug-loaded human low density lipoproteins. Journal of Pharmaceutical Sciences 84: 139 – 147.

Wilmar S. (1960). Pharmacolozical study of compouad isolated from crataegus Arzneittel-Forsch. The Traditional Chinese Medicine 60 – 61.

Yeh G. C., Ochipinti S. J., Cowan K. H., Chabner B. A., and Myers C. E. (1987). Adriamycin resistance in human tumor cells associated with marked alterations in the regulation of hexonse monophosphate shunt and its response to oxidant stress. Cancer Research 47: 5994 – 5999.

Zheleznova E. E., Markham P., Edgar R., Bibi E., Neyfakh A. A., and Brennan R. G. (2000). A structure-based mechanism for drug binding by multidrug transporters. Trends in Biotechnology 25: 39- 43.

Zouhair F. S., Lindsey S., and Hayes K. C. (1987). Taurine enhances low density lipoprotein binding internalization and degradation by cultured HepG2 cells. The Journal of Biological Chemistry 262: 6069 – 6073.